



**Guilherme de Abreu
Jeremias**

**O papel evolutivo dos mecanismos epigenéticos: a
adaptação de organismos dulçaquícolas às
alterações climáticas**

**The evolutionary role of epigenetic mechanisms: the
adaptation of freshwater organisms to climate
change**

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Joana Luísa Pereira e do Doutor Sérgio Miguel Marques, Investigadores em Pós-Doutoramento, e do Professor Doutor Fernando Gonçalves, Professor Associado com Agregação, todos do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

Aos meus pais. Aos mais próximos. Obrigado.

*“Não estando disposto a esperar que a humanidade venha
alguma vez a ser melhor,
Jeremias escolheu o seu lugar do lado de fora”*

Jeremias, o Fora-da-Lei, Jorge Palma

o júri

presidente

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palavras-chave

Ecossistemas de água doce, biodiversidade, alterações climáticas, salinidade, evolução, adaptação, epigenética, fenótipo, efeitos transgeracionais, *Daphnia magna*, conservação

resumo

A água doce é um dos recursos mais preciosos para a humanidade. No entanto, para além de serem alvo de significativas pressões antropogénicas, os sistemas dulçaquícolas enfrentam diversas transformações impostas pelas alterações climáticas. Em particular, as alterações climáticas são, hoje em dia, reconhecidas como uma ameaça séria para os ecossistemas dulçaquícolas, sendo que, à escala global, a biodiversidade de água doce está em rápido declínio.

Nos últimos anos, a epigenética tem ganho importância em diversos campos científicos, como a saúde humana e a biologia do desenvolvimento. De facto, os mecanismos epigenéticos regulam o aparecimento de novos fenótipos, estando comprovado que os fenótipos determinados epigeneticamente podem ser transmitidos ao longo de sucessivas gerações. Assim, a epigenética é um tema promissor mas largamente inexplorado num grande número de áreas científicas.

Procurando avaliar a resiliência das espécies dulçaquícolas para lidar com as transformações ambientais impostas pelas alterações climáticas, este trabalho teve como principais objetivos: i) rever os impactos das alterações climáticas nos ecossistemas dulçaquícolas, explorando a relação entre os mecanismos epigenéticos e a adaptação de diversos grupos de organismos a este fenómeno; ii) avaliar a potencial importância dos mecanismos epigenéticos na adaptação de *Daphnia magna* a um aumento na salinidade das águas.

Uma extensa revisão da literatura foi efetuada, tendo sido concluído que as alterações climáticas estão a alterar a composição, biodiversidade e funcionamento dos ecossistemas dulçaquícolas. Adicionalmente, este trabalho evidenciou que, no contexto das alterações climáticas, o aumento da temperatura e/ou salinidade da água são as principais ameaças para a biodiversidade dulçaquícola, sendo que os mecanismos epigenéticos podem contribuir decisivamente para a adaptação de diferentes grupos taxonómicos, tanto a curto como a longo prazo, às flutuações e transformações ambientais provocadas pelas alterações climáticas.

Na abordagem do segundo objetivo desta dissertação, a exposição de uma geração de *Daphnia magna* a um aumento de salinidade na água induziu uma adaptação molecular, ao nível epigenético, ao ambiente hipertónico. Para alguns genes, esta adaptação epigenética foi transferida ao longo de três sucessivas gerações, nenhuma das quais expostas ao agente de *stress* a que esteve exposta a geração parental.

Globalmente, este trabalho suporta a ideia de que os mecanismos epigenéticos são um tema promissor nas áreas da ecotoxicologia aquática e biologia evolutiva, bem como na interface entre estas duas áreas de investigação.

keywords

Freshwater ecosystems, biodiversity, climate change, salinity, evolution, adaptation, epigenetics, phenotype, transgenerational effects, *Daphnia magna*, conservation

abstract

Freshwater is a precious resource for humankind. Nevertheless, freshwater systems face not only significant anthropogenic pressure but also numerous transformations induced by climate change. Nowadays, climate change is recognized as a serious threat to freshwater ecosystems and, on a global scale, freshwater biodiversity is in rapid decline.

In the last few years, epigenetics has been gaining importance in several scientific fields, such as human health and developmental biology. In fact, epigenetic mechanisms can influence phenotype determination and epigenetically acquired phenotypes are known to be inherited across several generations. Therefore, epigenetics is a promising but unexplored topic in a significant number of scientific fields.

Taking into account that the global aim of this work was to explore the resilience of freshwater species to cope with the environmental transformations induced by climate change, its main specific objectives were: i) to review the impacts of climate change on freshwater ecosystems, and explore the link between epigenetic mechanisms and the adaptation of different species to the associated phenomena; ii) to assess the relevance of epigenetic mechanisms in the adaptation of *Daphnia magna* to an increase in water salinity.

An extensive review was made leading to the conclusion that climate change is transforming the composition, biodiversity and functioning of numerous freshwater ecosystems. Additionally, this study highlighted that, in the context of climate change, the rise in water temperature and/or salinity are the major threats to freshwater biodiversity, while epigenetic mechanisms can have a decisive role in constraining both short- and long-term adaptation of different taxonomic groups to the environmental transformations induced by climate change.

As the second objective was tackled, the exposure of one generation of *Daphnia magna* to increased salinity showed that there is a molecular adaptation to the hyperosmotic environment at the epigenetic level. For some genes, this epigenetic adaptation was transferred across three successive generations, none of which exposed to the stressor challenging the parental generation.

Generally, this work supports the idea that epigenetic mechanisms are a promising topic in the fields of aquatic toxicology and evolutionary biology, as well as in research undertaken in their intersection.

INDEX

Chapter 1 - General introduction and objectives	3
1.1. Status of the World's Freshwater Systems	3
1.2. Climate Change	6
1.3. Impacts of Climate Change on Freshwater Ecosystems.....	9
1.3.1. Salinity Rising Due to Climate Change and its Implications for Society	10
1.3.2. Effects of Salinity Rising on Freshwater Ecosystems	12
1.4. Physiology of <i>Daphnia</i>	16
1.4.1. Osmotic and Ionic Regulation	16
1.4.2. The role of Na ⁺ /K ⁺ -ATPase in Osmoregulation	18
1.5. Epigenetics	22
1.5.1. Epigenetic Mechanisms	22
1.5.2. <i>Daphnia</i> Epigenetics	23
1.5.3. Transgenerational Epigenetic Inheritance	25
1.7. Objectives and Structure of the Dissertation.....	27
1.8. References.....	29
Chapter 2 - The role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems: a review	41
2.1. Abstract	41
2.2. Introduction	41
2.3. Condition of World's Freshwater: Drivers of Transformation	42
2.4. Impacts of Climate Change on Freshwater Ecosystems	43
2.5. Epigenetic Mechanisms and their Evolutionary Role	48
2.6. Epigenetic Adaptation to Climate Change	52

2.7. Conclusions and Perspectives for Future Research	55
2.8. References	58
Chapter 3 - Transgenerational effects of salinity stress in <i>Daphnia magna</i>: the role of epigenetic mechanisms	67
3.1. Abstract	67
3.2. Introduction	67
3.3. Material and Methods	71
3.3.1. <i>Daphnia</i> culturing	71
3.3.2. Exposure and sampling	72
3.3.3. Reduced representation bisulfite sequencing	73
3.3.4. Bioinformatic analysis	74
3.3.5. Statistical analysis	74
3.4. Results	75
3.5. Discussion	77
3.6. References	82
Chapter 4 - General discussion and final remarks	91

Chapter 1 - General Introduction and Objectives

1.1. Status of the World's Freshwater Systems

Hydrosphere is composed by all the available water (liquid, soil and gas) in the atmosphere, on the surface of the Earth and in the Earth's crust. Its volume corresponds approximately to $1.386 \times 10^6 \text{ Km}^3$ but freshwater comprises only 2.5% of it. In terms of freshwater distribution, 68.7% is perennially frozen and 29.9% is groundwater. Moreover, only 0.26% of the Earth's freshwater can be found in lakes, reservoirs and rivers (Carpenter, Stanley, & Vander Zanden, 2011).

Thus, freshwater is the most precious resource for humankind. Besides humans' basic consumption needs, freshwater plays a key role in industrial processes, particularly in food, energy and fibre production, and it is vital as a recipient to the dilution and degradation of pollutants (Carpenter et al., 2011; Drechsel, Qadir, & Wichelns, 2015; Rockström, Gordon, Folke, Falkenmark, & Engwall, 1999; WWAP, 2014). Consequently, freshwater scarcity has been set forward has one of the most dangerous threats for human societies (Carpenter et al., 2011; Mekonnen & Hoekstra, 2016; Millennium Ecosystem Assessment, 2005).

Nowadays, there are still 748 million people who cannot have access to a convenient source of water and an estimated 2.4 billion people live in water-stressed environments. Moreover, as Mekonnen & Hoekstra (2016) unveiled, two thirds of the world's population live in regions that face water scarcity at least for one month a year (Figure 1). Worldwide, human demand for freshwater currently approaches or exceeds its supply, and the provision of freshwater to populations is only achieved following disruptive exploitation in numerous terrestrial and aquatic ecosystems (Carpenter et al., 2011; Jackson et al., 2001; Guy Woodward, Perkins, & Brown, 2010).

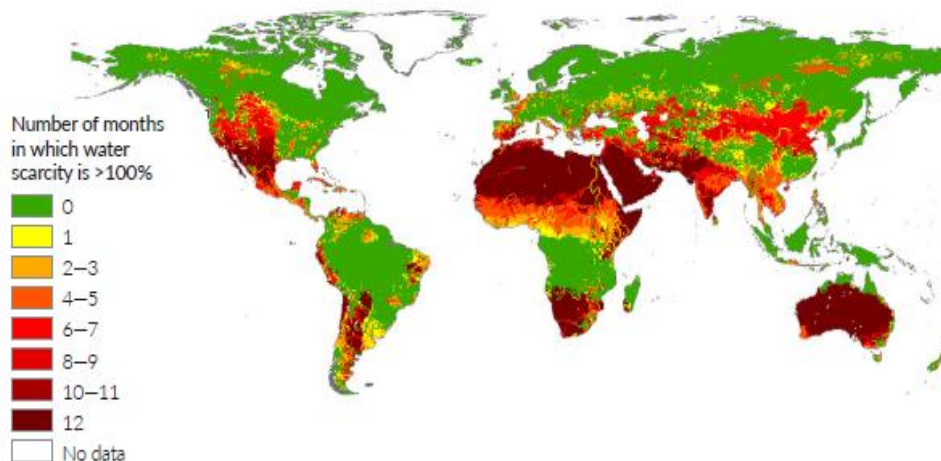


Figure 1 - Number of months per year in which usable freshwater scarcity exceeds 1.0 – which is the ratio between the footprint (volume of fresh surface water and groundwater withdrawn, and not returned due to evaporation or human use) and the total freshwater availability - at 30 × 30 arc min resolution (1996-2005). Source: Mekonnen & Hoekstra (2016).

The agricultural sector is currently responsible for 70% of all freshwater consumption in the world (Rosegrant, Ringler, & Zhu, 2009; WWAP, 2017), as shown in Figure 2. In the future, significant increases in freshwater consumption are expected for industry and energy production, as several energy sources such as nuclear, hydroelectric and thermal require water for production, safety and transport (WWAP, 2014, 2017). These trends on freshwater consumption are closely related to the projections of Earth's population growth. For example, the population of the Asia-Pacific region is currently rising and it is expected to reach 5 billion in 2050 (WWAP, 2017). Consequently, the higher demand for freshwater will result in some freshwater systems which will be further explored up to their limits (Dudgeon, 2000; WWAP, 2017).

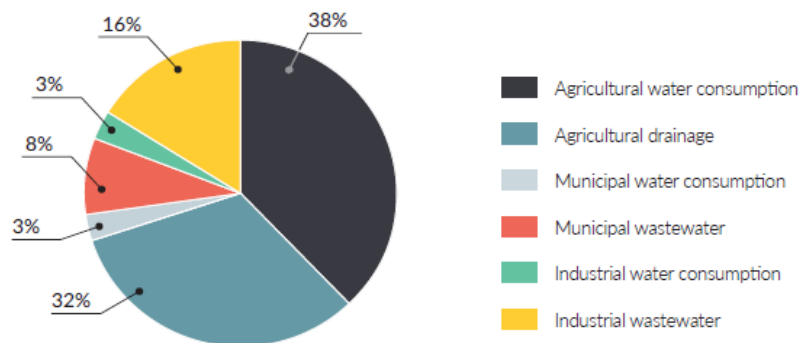


Figure 2 - Global consumption and wastewater production by major water use sector. Data refers to 2010. Source: WWAP (2017).

In addition to direct exploitation, freshwater systems face various other anthropogenic pressures. For instance, several authors highlighted the impacts of land use in these systems (Delgado, Rosegrant, Steinfeld, Ehui, & Courbois, 1999; Foley, 2005; Ramankutty, Evan, Monfreda, & Foley, 2008). Besides, the drainage of chemicals from agricultural and urban areas and the discharge of contaminated waters from e.g. mining, medical and industrial activities lead to an increase in the quantity of exogenous chemicals such as organic compounds, heavy metals, acids and alkalis which are released and spread throughout freshwater ecosystems (Carpenter et al., 2011; Durance & Ormerod, 2009; Guy Woodward, Perkins, et al., 2010). Several of these contaminants are reportedly toxic to aquatic organisms and humans (Corcoran, Nellemann, Baker, Bos, & Osborn, 2010; Drechsel et al., 2015; WWAP, 2017). Furthermore, wastewaters are also another important source of freshwater contamination (Figure 3). An estimated 80% of the world's wastewaters (values can reach 95% in some developing countries) are discharged into the environment without treatment (WWAP, 2017). Remarkably, the rise in water scarcity in some parts of the world has led to a generalized, although still limited recognition of the importance of wastewater collection, treatment and reuse (Corcoran et al., 2010; WWAP, 2017).

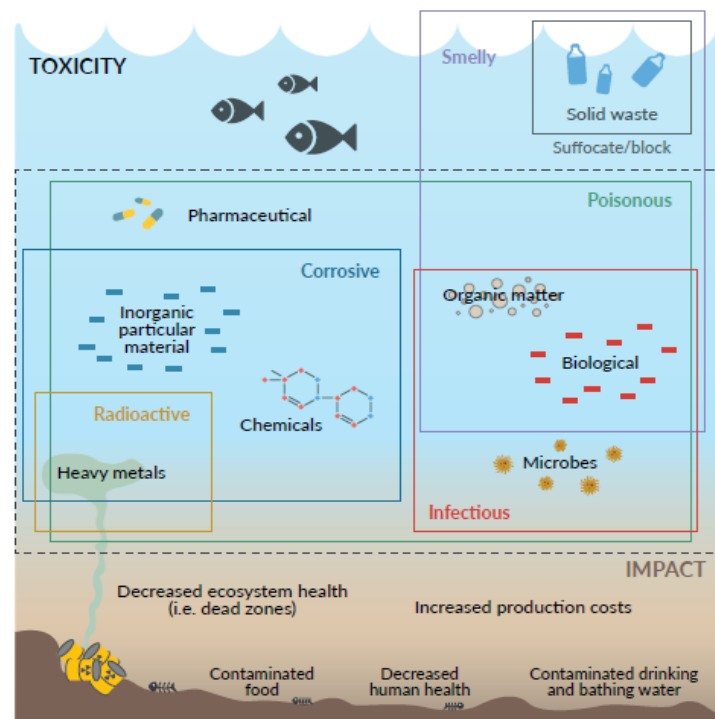


Figure 3 - Wastewater components and their effects. Source: WWAP (2017).

Besides several anthropogenic impacts, climate change has been pointed out as another important driver of freshwater transformation (Carpenter et al., 2011; Michener, Blood, Bildstein, Brinson, & Gardner, 1997; Guy Woodward, Perkins, et al., 2010). It is a serious issue that has risen as a consequence of human activities (IPCC, 2007; Schewe et al., 2014), and projections suggest that climate change will further cause profound alterations in freshwater systems within a few years (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Guy Woodward, Perkins, et al., 2010). In fact, climate change impacts can already be seen worldwide and their magnitude made them a noteworthy subject and a major topic within political agendas (IPCC, 2007, 2014).

Freshwater systems are among the most altered systems on Earth, although their protection and conservation is critical to the well-being of future generations and the preservation of biodiversity (Carpenter et al., 2011; Dudgeon et al., 2006). In order to contextualise the urgent need for an improved freshwater ecosystems management, part of the United Nations World Water Development Report (2017) can be quoted: *“In a world where demands for freshwater are ever growing, and where limited water resources are increasingly stressed by over-abstraction, pollution and climate change, neglecting the opportunities arising from improved wastewater management is nothing less than unthinkable”*.

1.2. Climate Change

The climate of Earth has been constantly changing throughout time. As scientific data shows, there have been 7 cycles of glacial advance and retreat in the last 650,000 years. The last ice age occurred 7,000 years ago and since then the planet has entered in the so-called modern climate era. While these climatic fluctuations were determined by small variations in the orbit of the Earth (which changed the quantity of solar energy that the planet receives), human activities are responsible for the recent alteration of the Earth's climate (NASA, 2008).

Humans have been drastically changing their way of living and, since the industrial revolution in particular, there has been a huge rise in the quantity of greenhouse gases released to the atmosphere. This increase has been particularly evident from the mid-20th century onwards (IPCC, 2007). Despite the

contribution of some natural processes to the release of these gases, the four principal ones - carbon dioxide (CO₂), methane (CH₄), nitrous oxide (N₂O) and halocarbons (a group of gases containing fluorine, chlorine and bromine) - have significantly risen their concentration in the atmosphere as a consequence of human activities (IPCC, 2007; National Research Council, 2006; Vitousek et al., 1997). Specifically, methane levels increased as a result of agriculture practices and natural gas distribution while nitrous oxide levels risen mostly due to the use of fertilizers and fossil fuel burning (Milich, 1999; Vitousek et al., 1997). On the other hand, halocarbon gas increased following its use as a refrigeration agent and in other industrial processes (NASA, 2008; Ramanathan, Cicerone, Singh, & Kiehl, 1985). Carbon dioxide atmospheric concentrations are those experiencing the most dramatic increase (Figure 4) and this increase was mainly determined by the burning of fossil fuels for e.g. transportation, building refrigeration and in the manufacture of goods (Crowley, 2001; IPCC, 2007; Ramanathan et al., 1985; Sabine, 2004).

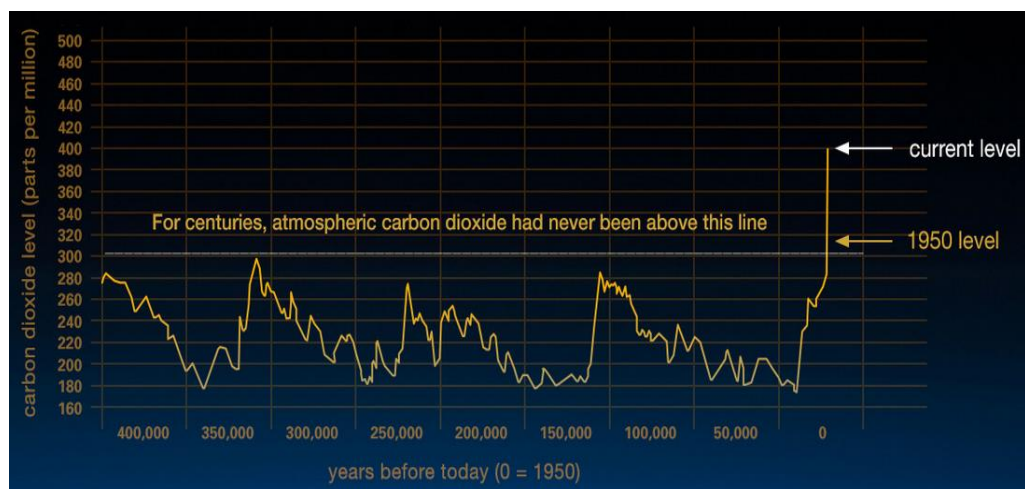


Figure 4 - Atmospheric carbon dioxide levels (parts per million) over time. The graph is based on the comparison of atmospheric samples taken from ice cores and more recent direct measurements. Adapted from NASA (2008). Link: <https://climate.nasa.gov/evidence/>.

The Earth holds a complex energy balance. In fact, part of the radiation emitted from the sun is reflected back to the space by clouds, atmospheric particles and bright surfaces, such as ice and snow on the ground. In addition, a percentage of the incoming solar radiation is absorbed in the atmosphere by dust,

atmospheric gases (including greenhouse gases) and other particles (Allen, 2009; Budyko, 1969; Trenberth, 2009). Therefore, as the concentration of greenhouse gases in the atmosphere rises, higher retention of incoming solar radiation occurs. As greenhouse gases have a heat-trapping nature, the increasing atmospheric concentrations of these gases results in higher temperatures on the Earth's surface (Allen, 2009; IPCC, 2007, 2014; Kunkel et al., 2013; National Research Council, 2006). The scientific evidences revealing that Earth's climate responds to changes in greenhouse gas levels are many, impressed in e.g. ice cores (Figure 5) from Greenland, Antarctica and tropical mountain glaciers (Alley, 2000; Derksen & Brown, 2012), as well as ocean sediments, coral reefs, sedimentary rocks and tree rings. These palaeoclimate evidences indicate that the pace of warming that the Earth experiences today is roughly ten times faster than it was in previous normal warming periods related to the ice-age-recovery (Alley, 2000; Mengel et al., 2016; NASA, 2008; National Research Council, 2006).



Figure 5 - Experimental drilling on the Greenland Ice Cap, summer of 2005. Source: NASA (2008). Link: https://earthobservatory.nasa.gov/Features/Paleoclimatology_IceCores/.

The consequences of climate change can already be seen worldwide. Among these, some remarkable ones are the rise in the global surface temperature, the rise of sea level, the shrinking of ice sheets, the decline of Arctic sea ice, the retreat of glaciers, the acidification of oceans and the higher frequency of extreme events, such as droughts and floods (Alley, 2000; Derksen & Brown, 2012; IPCC, 2007, 2014; Mengel et al., 2016; Michener et al., 1997; NASA, 2008).

All these are frighteningly affecting human existence and significantly pressuring entire ecosystems.

1.3. Impacts of Climate Change on Freshwater Ecosystems

Climate change has provoked significant alterations in several climate trends (IPCC, 2007; Michener et al., 1997; Rahmstorf, 2007), at an unprecedented speed (Guy Woodward, Dybkjær, et al., 2010). Therefore, climate change has been touted as the greatest emerging threat to global biodiversity and to the functioning of ecosystems (Bastawrous & Hennig, 2014; Dudgeon et al., 2006; Guy Woodward, Perkins, et al., 2010).

Freshwater ecosystems present several features that make them particularly vulnerable to climate change. For example, freshwater ecosystems are relatively isolated and physically fragmented within large terrestrial landscapes (Dudgeon et al., 2006; Guy Woodward, Perkins, et al., 2010). Thus, freshwater species cannot easily disperse while the environment is changing (Dudgeon et al., 2006; Guy Woodward, Perkins, et al., 2010). Moreover, freshwater ecosystems face a myriad of anthropogenic pressures while they are heavily explored for goods and services (Carpenter et al., 2011; Foley, 2005; Malmqvist et al., 2008), and despite a small percentage of the Earth surface is covered by freshwater ecosystems (approximately 0.8%), they have been estimated to support almost 6% of all described species (Dudgeon et al., 2006). Consequently, Woodward, Perkins, & Brown, (2010) have claimed that *“freshwater biodiversity is disproportionately at risk on a global scale”*.

The impacts of climate change have already been recorded in freshwater systems standing in higher latitudes and altitudes, since these regions have been experiencing higher rates of warming (IPCC, 2007; Guy Woodward, Perkins, et al., 2010). These particular systems have been considered ‘sentinel systems’, as they can provide early warning evidences on the impacts of climate change in freshwater systems (Layer, Hildrew, Monteith, & Woodward, 2010; Guy Woodward, Dybkjær, et al., 2010). In fact, several Arctic lakes and ponds have been recording regime shifts in their biological communities (Smol et al., 2005), which has been linked to long-term warming. Warmer temperatures affect

freshwater systems by decreasing the duration of the ice season, as they influence the melting of glaciers, permafrost and other ice sheets. Moreover, a warmer climate also contributes to changes in evaporation and precipitation ratios (Carpenter et al., 2011; Oechel et al., 2000; Smol et al., 2005). All these factors constitute important causes of limnological changes, provided they provoke transformations in the seasonality and magnitude of hydrologic flows (Carpenter et al., 2011; Smol et al., 2005).

Another climate change-related driver of freshwater transformation is the rise in salinity (Chen & Zong, 1999; Mengel et al., 2016; Michener et al., 1997; Mimura, 2013; Rahmstorf, 2010). Mainly provoked by sea level rising, the rise in salinity in freshwater ecosystems has severe impacts on freshwater biodiversity, as salinity affects the survival and reproduction of freshwater species (Carpenter et al., 2011; Martínez-Jerónimo & Martínez-Jerónimo, 2007; Mimura, 2013; Nielsen, Brock, Rees, & Baldwin, 2003; Guy Woodward, Perkins, et al., 2010).

In summary, climate change is transforming the composition, biodiversity and functioning of freshwater ecosystems (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Durance & Ormerod, 2009; Williams, 1987; Guy Woodward, Perkins, et al., 2010). A deep understanding on the capacity of freshwater organisms to cope with environmental changes is critical to better picture the resilience of freshwater ecosystems under related environmental pressure and hence to a conscientious development of effective protection/management actions towards the conservation of these valuable resources (Bastawrous & Hennig, 2014; Dudgeon et al., 2006; Guy Woodward, Perkins, et al., 2010).

1.3.1. Salinity Rising Due to Climate Change and its Implications for Society

Numerous studies have tried to understand and predict the implications of climate change for human societies and ecosystems (Bastawrous & Hennig, 2014; IPCC, 2007; Vineis, Chan, & Khan, 2011; Vörösmarty, 2000; Guy Woodward, Perkins, et al., 2010). Amongst them, several highlighted the problems related to the rising of salinity in freshwaters (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Schallenberg, Hall, & Burns, 2003; Vineis et al., 2011; Guy Woodward, Perkins, et

al., 2010). This salinity increase is mostly driven by two phenomena: sea level rise and evaporation (Schallenberg et al., 2003; Sumner & Belaine, 2005). Higher evaporation rates lead to a reduction in the total volume of the watersheds, therefore rising waterborne salt concentration. In parallel, sea level rise (Figure 6) intensifies the intrusion of sea water into freshwaters, provoking a rise in salinity in these latter ecosystems (Mengel et al., 2016; Mimura, 2013).

Sea level is influenced by a great number of factors. The most important ones leading to increased sea levels are the added water from land-based ice melting and the thermal expansion of the oceans (water expands as it warms) (Mimura, 2013). As provoked by climate change, sea level started to rise during the 19th century and rose around 20 centimetres in the last century (IPCC, 2007; Mimura, 2013). Despite several efforts to predict future sea level trends, there is a clear lack of scientific agreement about the projections already made. For example, the Intergovernmental Panel on Climate Change (IPCC) projected global sea level to rise 18 to 59 centimetres from 1990 to the 2090s (IPCC, 2007). However, several authors have been showing that physical models used by IPCC have several limitations (Mimura, 2013; Rahmstorf, 2007, 2010), and predicted much higher sea level values in the future. As Rahmstorf (2010) summed-up, “A number of recent studies taking the semi-empirical approach have predicted much higher sea level rise for the twenty-first century than the IPCC, exceeding one metre if greenhouse gas emissions continue to escalate”.



Figure 6 - Changes in sea level since 1993 (satellite observations). Source: NASA (2008). Link: <https://climate.nasa.gov/vital-signs/sea-level/>.

Considering the projections for sea level rise, humans and freshwater ecosystems will face additional pressure, particularly in low-land coastal zones (continuous areas along the coast recording altitudes lower than 10 meters above sea level) (McGranahan, Balk, & Anderson, 2007; Mimura, 2013). Despite covering only 2% of the Earth's surface, they hold 10% of the world population (McGranahan et al., 2007). In addition, the impacts of sea level rise will be felt in other flooding-vulnerable areas, such as delta regions and small islands (McGranahan et al., 2007; Mimura, 2009), which are heavily populated and prone to coastal erosion, respectively. Furthermore, sea water intrusion will contaminate coastal aquifers, tidal waterways and freshwaters, such as lakes and other reservoirs (Carpenter et al., 2011; Chen & Zong, 1999; Michener et al., 1997; Vineis et al., 2011; Guy Woodward, Perkins, et al., 2010).

In summary, the increase of salinity levels in freshwater systems has significant and complex impacts on the natural environment and society. Actually, salinity promotes the alteration of the physical, chemical and biological features of aquatic systems (Cañedo-Argüelles et al., 2013; Carpenter, Kitchell, & Hodgson, 1985; Carpenter et al., 2011; Nielsen et al., 2003; Guy Woodward, Perkins, et al., 2010).

1.3.2. Effects of Salinity Rising on Freshwater Ecosystems

Salinity is a property of all natural waters and it refers to the total concentration of dissolved inorganic ions (Williams & Sherwood, 1994). Still, salinity is mostly determined by the dilution of Na^+ , Ca^{2+} , Mg^{2+} , K^+ , Cl^- , SO_4^{2-} , CO_3^{2-} and HCO_3^- (Williams, 1987).

Salinity influences the distribution of aquatic biota and species are commonly aggregated according to their salinity preferences into three major groups collecting freshwater fauna, brackish water fauna and marine fauna (Aladin & Potts, 1995; Williams, 1987). The salinization of freshwaters constitutes an acute form of environmental perturbation (Millennium Ecosystem Assessment, 2005; Schallenberg et al., 2003; Williams, 1987). In fact, once the salt concentration increases in a freshwater ecosystem, organisms can only survive and reproduce if they adapt themselves to the new environment or if they

behaviourally can avoid the stressor (Berg et al., 2010; Nielsen et al., 2003). Since freshwater ecosystems are physically fragmented within large terrestrial landscapes, species with limited dispersal abilities must adapt or they will perish (Dudgeon et al., 2006; Eros & Campbell Grant, 2015; Guy Woodward, Perkins, et al., 2010). Therefore, even relatively small changes in salinity can have an impact on freshwater and brackish ecosystems by depleting biodiversity and by changing their dynamics and functioning (Nielsen et al., 2003; Schallenberg et al., 2003).

Freshwater ecosystems have a typical trophic structure which is constituted by four main functional groups: phytoplankton, zooplankton, vertebrate and invertebrate planktivores and top predators (piscivorous fishes) (Leibold, Chase, Shurin, & Downing, 1997). Zooplankton is composed by three essential groups of organisms: rotifers, copepods and cladocerans, these latter being pointed out as the most relevant for freshwater ecosystems (Lampert, 2006; Leibold et al., 1997; Wetzel, 1993). Among cladocerans (order Cladocera), those belonging the genus *Daphnia* have been highlighted because of their larger size. In fact, this characteristic allows them a higher capacity of suppressing phytoplankton (Gliwicz & Guisande, 1992) and makes them the preferential (and/or easier) food source for visual predators, such as fish (Hall et al., 1976). By playing this central role (Figure 7), daphnids are major zooplankton actors in the recycling of nutrients, therefore markedly influencing the primary productivity of freshwater ecosystems (Carpenter et al., 1985; Hall et al., 1976). Indeed, *Daphnia species* are major contributors to the decisive ecological role of zooplankton supporting the structure and function of freshwater ecosystems (Carpenter et al., 1985; Nielsen et al., 2003).

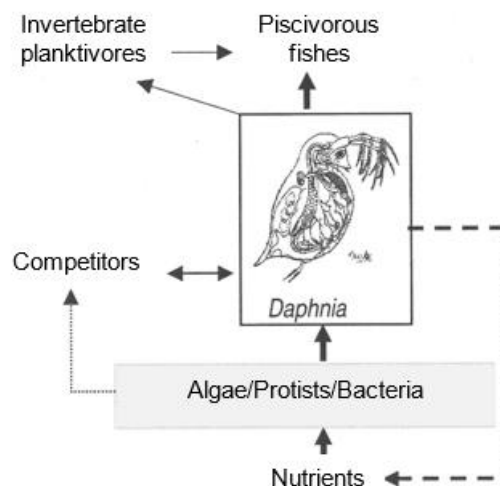


Figure 7 - Trophic interactions and food web structure in a pelagic, freshwater ecosystem. Adapted from Pereira (2008).

Zooplankters can be severely affected by salinity stress, through the impairment of their growth, reproduction and survival (Bailey et al., 2004; Hall & Burns, 2003; Jeppesen et al., 1994; Schallenberg et al., 2003). Once their survival and life-history are affected, population structure and dynamics changes and higher trophic levels can consequently be severely harmed along with the capacity of the system to control primary productivity, ultimately putting the entire ecosystem at risk (Brucet et al., 2010; Cañedo-Argüelles et al., 2013; Hall & Burns, 2003; Lampert, 2006). Although their common sensitivity to higher salt concentrations, different groups of organisms within zooplankton present distinct physiological tolerances to several ionic components of the medium (Boronat, Miracle, & Armengol, 2001; Bos, Cumming, Watters, & Smol, 1996). For instance, the animals of the Cladocera order have been considered extremely good indicators of salinity changes (Boronat et al., 2001) and it has been proved that they present differential halotolerance, ranging from fresh to brackish water levels (Aladin & Potts, 1995; Boronat et al., 2001; Bos et al., 1996; Lockwood, 1962).

Daphnia magna is a species of cladocerans whose specific patterns of tolerance to salinity are remarkably different compared to other *Daphnia* species. *D. magna* has an original holarctic distribution but it is also able to occupy arid climatic regions (e.g. Africa countries), tolerating a broad range of environmental

conditions (Alonso, 1996; Benzie, 2005). As far as salinity tolerance is concerned, *D. magna* is deemed euryhaline and it is able to live not only in fresh and brackish waters, but also in athalassohaline waters, surviving to salt ranges of 3.8 to 38 g.L⁻¹ (Alonso, 1996; Boronat et al., 2001). Therefore, they are able to live in temporary pools and ponds where fluctuations of salinity are commonly higher than in permanent larger ponds and lakes. In fact, *D. magna* is one of the most halotolerant organisms from the *Daphnia* genus. For example, Gonçalves et al. (2007) addressed the tolerance of *Daphnia magna* and *Daphnia longispina* to salinity in terms of immobilisation (acute toxicity) and life history (chronic toxicity), finding that NaCl acute and chronic toxicity to *D. magna* (EC₅₀ values of 5.9 g.L⁻¹ and 5.0 g.L⁻¹, respectively) was about two-fold lower compared to *D. longispina* (EC₅₀ values of 2.9 g.L⁻¹ and 2.2 g.L⁻¹, respectively). Another conclusion of the study was that *D. magna* reproduces and lives well at salinity concentrations up to 4.55 g.L⁻¹, a reference similar to the one obtained by Schuytema, Nebeker, and Stutzman (1997), who found that *D. magna* reproduces and survives normally at salinity concentrations up to 4 g.L⁻¹. Consistently, other authors pointed out that as salinity increases, freshwater communities experience a quick composition shift into the prevalence of species more tolerant to salt, including *D. magna*, while the least tolerant ones tend to suffer deleterious effects (Brucet et al., 2010; Horrigan, Choy, Marshall, & Recknagel, 2005; Nielsen et al., 2003).

Interestingly, *D. magna* populations show remarkable clonal variation regarding salt tolerance, with some clones being able to live only in freshwater and others tolerating in brackish waters (Martínez-Jerónimo & Martínez-Jerónimo, 2007; Teschner, 1995; Weider & Hebert, 1987). However, salinity sensitivity is not restricted to freshwater clones: both freshwater and brackish clones of *D. magna* showed the same negative effects on development and reproduction when grown at 5‰ (Teschner, 1995). So, despite the ability of *D. magna* to survive in a broad range of salt concentrations, higher concentrations usually have deleterious effects on its reproduction and consequently on the population growth of this species (Arnér & Koivisto, 1993; Cowgill & Milazzo, 1991; Ghazy, Habashy, Kossa, & Mohammady, 2009; Martínez-Jerónimo & Martínez-Jerónimo, 2007). Therefore, it may take a long period of time until daphnids fully adapt to higher

salinity conditions (Ghazy et al., 2009; Martínez-Jerónimo & Martínez-Jerónimo, 2007).

By the key role of *Daphnia* species in freshwater ecosystems, they are widely seen as valuable indicators of the ecosystem responses to environmental change (e.g. Colbourne et al., 2011). Moreover, *Daphnia* organisms are easy to handle with and it is simple to maintain them under laboratory conditions, profiting from their rapid and highly productive life cycle (Baudo, 1987; Lampert, 2006). Due to all these factors, *Daphnia* species have been established as model organisms in several fields of science, such as ecology, ecotoxicology and evolutionary biology (Baudo, 1987; Deng & Lynch, 1996; Hebert, 1987; Lampert, 2006).

1.4. Physiology of *Daphnia*

1.4.1. Osmotic and Ionic Regulation

Freshwater species are hyperosmotic regulators, meaning that they keep their body fluids at higher salt concentrations than those prevailing in the external medium (Aladin & Potts, 1995; Lockwood, 1962). Osmoregulation is a key process for daphnids, as they need to counteract the natural, continuous ion loss to the water (Aladin & Potts, 1995; Lockwood, 1962; Lucu & Towle, 2003).

Daphnia have numerous osmoregulatory traits, some of these facing ontogenic changes during the reproduction cycle and development of daphnids. Under favourable conditions, *Daphnia* reproduce asexually by parthenogenesis for one to several generations. Under a parthenogenetic cycle, the offspring are genetically identical to the progenitors and the population consists of only females (Gabriel, 1982; Lynch & Gabriel, 1983). The parthenogenetic eggs are incubated in brood chambers which are located between the thorax and the dorsal carapace (Ebert, 2005) (Figure 8). The brood chamber is in direct contact with the external medium and the egg membrane is impermeable until the development of osmoregulatory organs in the emerging embryos (Aladin & Potts, 1995). In order to protect the developing embryo from salinity fluctuations, the “*osmolality of the embryonic fluid is isosmotic to the osmolality of the brood chamber fluid, which in turn is very similar to the osmolality of the haemolymph of the progenitor*” (Bianchini & Wood, 2008). When unfavourable environmental conditions arise,

Daphnia switches to sexual reproduction (Hobæk & Larsson, 1990; Innes & Singleton, 2000). In fact, the production of sexual eggs promotes genetic variation in the offspring (Hobæk & Larsson, 1990). These eggs are involved in a set of protective membranes (ephippium) and they are genetically and biochemically different from parthenogenetic eggs (Hebert, 1987; Pauwels, Stoks, Verbiest, & De Meester, 2007). Przylecki (1921a, 1921b) examined eggs from *D. magna* and found that over-wintering eggs bear higher salt content (4.2 g/L NaCl) than developing eggs (2.5 g/L NaCl). Therefore, over-wintering eggs are more resistant to environmental salinity fluctuations.

Osmoregulatory organs in embryos include the neck organ, whose function is largely assumed in later stages by epipodite cells (Aladin & Potts, 1995). The neck organ (also known as nuchal organ) contains mitochondria-rich cells to support salt uptake and the maxillary gland for water excretion (Aladin & Plotnikov I.S., 1985; Aladin & Potts, 1995; Lucu & Towle, 2003).



Figure 8 - Adult female of *Daphnia magna* with a clutch of freshly laid parthenogenetic eggs in its brood chamber. Source: Ebert (2005).

As powerful osmoregulators, daphnids actively uptake NaCl, which is essential to counteract the continuous ion loss (Bianchini & Wood, 2008; Lockwood, 1962; Lucu & Towle, 2003). Moreover, *Daphnia* clones have quantitatively differential sodium uptake kinetics (Havas, Hutchinson, & Likens, 1984; Potts & Fryer, 1979) and it has been proposed that these differences

represent physiological adaptations to different environments (Aladin & Potts, 1995).

The osmoregulatory mechanisms in *D. magna* are significantly different for adults and neonates. The mechanisms of whole-body Na^+ uptake and the concentration-dependent kinetics differ according to the life stage considered (Bianchini & Wood, 2008; Glover & Wood, 2005). Bianchini & Wood (2008) showed that adult daphnids have a lower maximum capacity of Na^+ transport on a mass-specific basis but a higher affinity for Na^+ compared to neonates. In addition, these authors highlighted that at the basolateral membrane of the salt-transporting epithelia of neonates, Na^+ ions are pumped from the cells to the extracellular fluid by a Na^+/K^+ -ATPase and a Na^+/Cl^- exchanger. In adults, the mechanisms are similar but a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter replaces the Na^+/Cl^- exchanger (Bianchini & Wood, 2008).

Actually, the sodium uptake rate influences the sensitivity of freshwater animals to ionoregulatory toxicants such as metals (Bianchini & Wood, 2002, 2003; Grosell, Nielsen, & Bianchini, 2002). These findings are particularly relevant to the study of osmoregulation in *D. magna*, since this species has been pointed out as the most sensitive aquatic species to waterborne copper and silver following either acute or chronic exposures (Bianchini & Wood, 2008; Grosell et al., 2002; Ratte, 1999). Furthermore, *“the mechanisms of acute and chronic toxicity of these metals are associated with an alteration of the whole-body Na^+ concentration as a consequence of a metal-induced inhibition of the whole-body Na^+/K^+ -ATPase activity”* (Bianchini & Wood, 2008). The inhibition of sodium uptake through the blockage of Na^+/K^+ -ATPase resembles the mechanism of metal toxicity described for freshwater fishes (Bianchini & Wood, 2003).

1.4.2. The role of Na^+/K^+ -ATPase in Osmoregulation

The sodium pump, Na^+/K^+ -ATPase is a membrane-bound protein that uses the hydrolysis of ATP to transport three sodium (Na^+) ions from the cytosol to the extracellular medium and two potassium (K^+) ions in the opposite direction. The electrochemical gradient that Na^+/K^+ -ATPase generates is crucial to maintain the osmotic balance of the cell, the resting membrane potential of most tissues and

the excitable properties of muscle and nerve cells (Kaplan, 2002; Therien & Blostein, 2000). Moreover, this electrochemical gradient is also essential for the transport of nutrients and amino acids into the cell (Cereijido, 2004; Kaplan, 2002; Kühlbrandt, 2004; Therien & Blostein, 2000).

The Na⁺/K⁺-ATPase is a heteromeric protein that is composed by a catalytic α -subunit and an accompanying β -subunit (Blanco & Mercer, 1998; Lingrel & Kuntzweiler, 1994) (Figure 9). The α -subunit is the largest, the most functional and it is also that responsible for ion transport (Shull, Lane, & Lingrel, 1986; Shull, Schwartz, & Lingrel, 1985). The β -subunit is responsible for the proper trafficking of the protein complex to the plasma membrane (Kaplan, 2002). An optional third subunit, the γ -subunit, may also be present (Geering, 2006) (Figure 9). It is the smallest subunit of the complex and its function is to modulate the kinetic characteristics of the pump, in a tissue-specific way (Crambert & Geering, 2003; Garty & Karlish, 2006; Geering, 2006). These three subunits have several isoforms, which are specific of different tissues and organisms (Blanco & Mercer, 1998; Garty & Karlish, 2006; Henriksen et al., 2013; Kaplan, 2002). Encoded by different genes, four isoforms of the α -subunit and three isoforms of β -subunit have been identified (Blanco, 2005; Henriksen et al., 2013; Sweadner, 1989).

Still, it has been demonstrated that there are minor differences in the amino acid sequences of the different subunit isoforms (Henriksen et al., 2013; Lucu & Towle, 2003; Suhail, 2010; Therien & Blostein, 2000). From an evolutionary point of view, highly conserved sequences indicate that Natural Selection continuously eliminated mutations in those sequences and the underlying reason for this high similarity appears to be the decisive role that the sodium pump plays in osmoregulation (Lucu & Towle, 2003; Masui, Furriel, Mantelatto, McNamara, & Leone, 2003; Sáez, Lozano, & Zaldívar-Riverón, 2009). As osmoregulation is an important process to all the forms of life on Earth, the sodium pump has suffered minor changes in its sequence in order to maintain its function (Sáez et al., 2009).

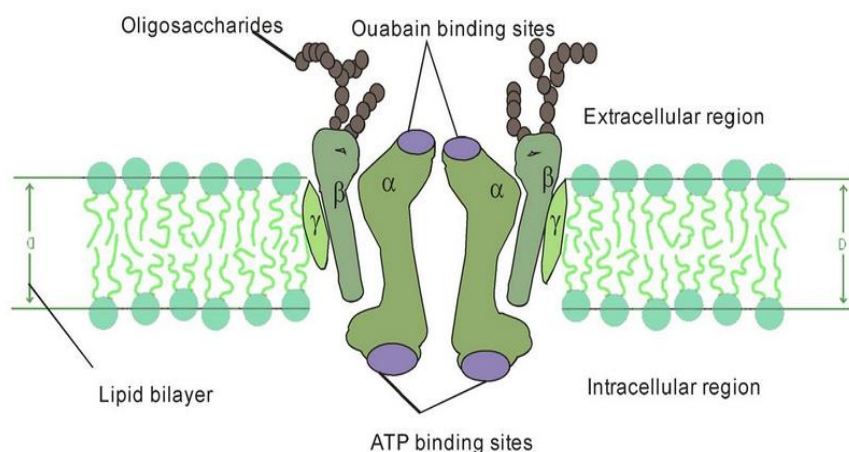


Figure 9 - Diagrammatic representation of Na⁺/K⁺-ATPase and its subunits (2α, 2β and 2γ-subunits) within a biomembrane. Source: Suhail (2010).

Several studies demonstrated that the Na⁺/K⁺-ATPase is the key molecule also for the osmoregulation of crustaceans in general (Henry, Lucu, Onken, & Weihrauch, 2012; Ituarte, López Mañanes, Spivak, & Anger, 2008; Leone et al., 2015; Lucu & Towle, 2003) and daphnids in particular (Bianchini & Wood, 2003, 2008; Latta, Weider, Colbourne, & Pfrender, 2012; Sáez et al., 2009). In other groups of animals, several works have also pointed out the biological importance of the sodium pump. For example, it has been established that the Na⁺/K⁺-ATPase plays a major role in the response of some fish species to salinity changes and in the acclimation of several species to freshwater environments (Lin, Tsai, and Lee 2004; Bystriansky and Schulte 2011).

As far as *Daphnia* species are concerned, several studies demonstrated that the sodium pump is also important to cope with different chemical substances (Bianchini & Wood, 2003, 2008; Latta et al., 2012). In terms of salinity stress, the Na⁺/K⁺-ATPase has been identified as the principal molecular feature involved in *Daphnia* responses though other molecular mechanisms may also play important roles (Bianchini & Wood, 2003, 2008; Henry et al., 2012). Sáez et al. (2009) studied the evolutionary history of Na⁺/K⁺-ATPases and pointed out that the major evidence on the relevance of a protein in osmoregulation is when its expression or activity is changed in the presence of different salt concentrations. Latta et al. (2012) investigated the effect of salinity in two genotypes of *D. pulex* that lived in environments with different salinity ranges, i.e. one of the genotypes lived in ponds

with salinity ranges of 0.11 to 0.58 g.L⁻¹ while the other inhabited ponds ranging from 0.60 to 9.95 g.L⁻¹. These authors were able to identify two copies of the Na⁺/K⁺-ATPase alpha subunit that were up-regulated in both genotypes as a response to increasing salinity. In addition, Sáez et al. (2009) performed a phylogenetic analysis with the available protein sequences (over 200) of the α -subunit of the sodium pump in different organisms. The authors found several genetic variations in the α -subunit of the Na⁺/K⁺-ATPase of *D. pulex*, which is consistent with an actual process of adaptation of *D. pulex* to different water salinities (Weider & Hebert, 1987). Actually, their idea is supported by other studies showing that the physiological response to environmental stressors can be constrained by the spatial and temporal regulation of the different isoforms of the Na⁺/K⁺-ATPase subunits (Escalante, Garcia-Saez, & Sastre, 1995; Jorgensen, Håkansson, & Karlsh, 2003; Li & Langhans, 2015).

Surprisingly, the specific mechanism that regulates the expression of Na⁺/K⁺-ATPase genes under different salt conditions wasn't identified yet. The spatial and temporal control of the sodium pump occurs at the transcriptional, post-transcriptional, translational and post-translational level (Capasso, Rivard, & Berl, 2005; Li & Langhans, 2015; Suhail, 2010). Moreover, the expression of Na⁺/K⁺-ATPase is transcriptionally regulated by hormones, growth factors, lipid mediators and other extracellular stimuli (Bajpai & Chaudhury, 1999; Billecocq et al., 1997; Cochrane et al., 2012). All these agents influence the binding of transcription factors to the promoter regions of the genes of the Na⁺/K⁺-ATPase subunits (Li & Langhans, 2015). In addition, several authors highlighted the potential importance of non-genetic factors in the gene regulation of Na⁺/K⁺-ATPase (Henriksen et al., 2013; Larsen et al., 2012; Li & Langhans, 2015; Selvakumar et al., 2014; Wang et al., 2013). Amongst these, early development acclimation, maternal factors and epigenetic factors have been pointed out as the best candidates for future research (Goetz & MacKenzie, 2008; Larsen et al., 2012; Vandegehuchte & Janssen, 2011). Remarkably, Selvakumar et al. (2014) recently showed that epigenetic modifications seem indeed to regulate the gene expression of the Na⁺/K⁺-ATPase in kidney cancer cells.

1.5. Epigenetics

1.5.1. Epigenetic Mechanisms

Epigenetics consists of changes in gene activity and expression without altering the underlying DNA sequence (Bernstein, Meissner, & Lander, 2007; Bird, 2007; Goldberg, Allis, & Bernstein, 2007). Epigenetic modifications are affected by environmental changes and are considered crucial for interpreting the genomes under the influence of physiological factors (Bernstein et al., 2007; Vandegehuchte & Janssen, 2011). Epigenetic mechanisms include the chemical modification of the cytosine residues of DNA (DNA methylation) and the modification of histones associated with DNA (protein modifications), and their response to environmental changes is achieved through the alteration of DNA accessibility and of chromatin structure (Bird, 2002, 2007; Goldberg et al., 2007; Kouzarides, 2007). In this way, these mechanisms can regulate gene expression and so they contribute to the determination of phenotypes. In addition, non-coding RNAs are included among epigenetic mechanisms. These RNAs are also capable of controlling gene expression, forming complex RNA regulatory networks of the genome (Long, Brady, & Benfey, 2009; Mattick & Makunin, 2006; Rinn & Chang, 2012).

DNA methylation is perhaps the most studied epigenetic mechanism. It consists of the addition of a methyl group (CH_3) to the 5' position of a cytosine ring, creating a 5-methylcytosine (m5C) (Figure 10). DNA methylation is catalysed by specific enzymes, namely methyltransferases (DNMTs). Some DNMTs are responsible for the maintenance of established patterns of DNA methylation while others catalyse *de novo* DNA methylation. Furthermore, DNA demethylation can also occur through the action of specific enzymes that are capable of demethylate m5Cs (Bird, 2002, 2007; Jaenisch & Bird, 2003; Zhong, Agha, & Baccarelli, 2016).

Epigenetics is considered one of the fastest-growing areas of Science and has been gaining importance in several fields mainly due to studies revealing the link between DNA methylation and several key molecular and cellular processes, such as transcriptional silencing, chromosome inactivation, transposable element regulation, development and tumorigenesis (Kingston, Tamkun, Baulcombe, & Dean, 2014; Morey & Avner, 2010; Sadikovic, Al-Romaih, Squire, & Zielenska, 2008; Sharma, Kelly, & Jones, 2009; Slotkin & Martienssen, 2007).

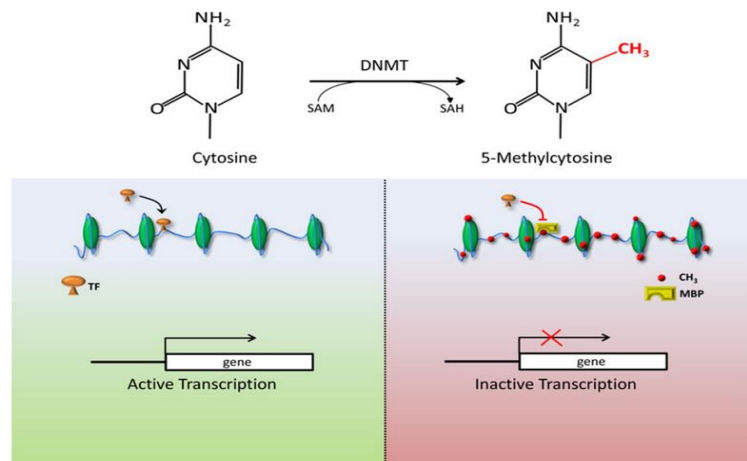


Figure 10 - DNA methylation and its role in the regulation of gene expression. Upper panel: Cytosine is methylated to 5-methylcytosine by DNA methyltransferase (DNMT); SAM (S-adenosylmethionine); SAH (S-adenosylhomocysteine). Lower panel: Binding of a methyl CpG-binding protein to methylated sequences prevents access to this sequence by transcription factors; MBP (methyl CpG-binding protein); TF (transcription factor). Source: Zhong et al. (2016).

1.5.2. *Daphnia* Epigenetics

Vandegehuchte et al. (2009) were the first studying *Daphnia* epigenetics. The authors found sequences homologous to human DNA methyltransferases (DNMT1, DNMT2 and DNMT3A) in the genome of *D. magna*. Furthermore, the analysis of two genomic fragments revealed low levels of methylation in *D. magna* (from 0.22% to 0.44% methylated CpG sites) (Vandegehuchte et al., 2009). These values are similar to methylation levels found in the *Drosophila melanogaster* genome (0.1% to 0.4%) (Lyko, Ramsahoye, & Jaenisch, 2003), but they are very low when compared to other species, as the percentage of methylated cytosines in the DNA of mammals and birds is 5%, 10% in fish and amphibians, up to 3% in insects and occasionally more than 30% in some plants (Field, Lyko, Mandrioli, & Prantera, 2004). Despite the relatively low levels of methylation found in the genome of *D. magna*, its potential importance in the regulation of growth and fertility of *Daphnia* species has been pointed out (Harris, Bartlett, & Lloyd, 2012; Robichaud, Sassine, Beaton, & Lloyd, 2012). Furthermore, it has also been shown that epigenetic modifications are present in the genome of *D. pulex* and that they are most likely involved in the regulation of gene expression (Strepetkaitė et al., 2015).

In an ecological and ecotoxicological context, the response of mammals to changes in the environment has been well characterized while studies inspecting

other animal groups are still scarce (Bernstein et al., 2007; Vandegehuchte & Janssen, 2014). Despite this scenario, *Daphnia* has already been set forward as an epigenetic model (Harris et al., 2012). The underlying reason for this touting is the reproductive strategy of these animals, which allows the establishment of parthenogenetical clones with the consequent possibility of eliminating genetic variability in experimental trials (Harris et al., 2012; Hebert, 1987). In addition, the draft genome sequence of *D. magna* is now available and there is a large quantity of ecological, ecotoxicological and evolutionary literature on *Daphnia* species that can facilitate the understanding of gene-environment interactions (Baudo, 1987; Harris et al., 2012; Lampert, 2006).

Few studies explored the epigenetic modifications in *Daphnia* following environmental challenge. Asselman et al. (2015) studied global cytosine methylation patterns following exposure of two different *D. magna* genotypes to 15 stressor gradients comprising metal burden, food quality, predation pressure, parasitism and water quality variation, including salinity increase. These authors proved that global cytosine methylation reflects genotype effects, environmental effects, and genotype \times environment effects. In addition, and also important in the context of the present dissertation, salinity was the single abiotic factor that significantly affected global DNA methylation levels in both clones tested. This study thus illustrates that changes in global DNA methylation are the result of complex interactions between genotype and environment, and that epigenetic effects may play a decisive role in constraining the response of populations to several changing environmental conditions, such as salinity.

In order to gain a better understanding of the relationship between DNA methylation and the response of *Daphnia* to different salinity conditions, it is important to specifically target the genes that are experiencing changes in their methylation status under different salinity levels. Na^+/K^+ -ATPase is the major molecular actor for proper osmoregulation in *Daphnia*, thus this is an interesting candidate gene group to look at in this context. Actually, it has been demonstrated that the expression of the genes of the Na^+/K^+ -ATPase subunits can be regulated at a transcriptional level through epigenetic mechanisms (Henriksen et al., 2013; Selvakumar et al., 2014; L. Wang et al., 2013). For instance, by studying kidney

carcinoma in humans, Selvakumar et al. (2014) were able to correlate reduced levels of the β -subunit protein (both in culture and in patients' tumour samples) to the hypermethylation in the promoter region for the human β -subunit gene. Furthermore, higher cytosine-guanine dinucleotides (CpGs) methylation was found in the first exon of the *Atp1a3* (one of the isoforms of the α -subunit) of pig liver cells and this was correlated to lower expression of this gene (Henriksen et al., 2013). Wang et al. (2013) consistently showed that the exposure of mice to manganese led to the hypermethylation of the *Atp1a3* promoter and that this methylation status provoked the downregulation of its transcript.

Despite notable efforts have been made, the role of epigenetic mechanisms in the regulation of *Daphnia* responses to environmental stimuli is still largely unexplored. However, as a different history of environmental exposure can entail different gene methylation patterns, it is undeniable that epigenetics opens interesting perspectives to the aquatic toxicology field (Asselman et al., 2017; Vandegehuchte & Janssen, 2011, 2014; Vandegehuchte, Lemière, & Janssen, 2009).

1.5.3. Transgenerational Epigenetic Inheritance

In general, epigenetic alterations are cleared and re-established at each generation, but some of them can be transferred through successive generations, even when the initial stress has disappeared (Bird, 2007; Daxinger & Whitelaw, 2010; Skinner, 2008; Vandegehuchte & Janssen, 2011) (Figure 11).

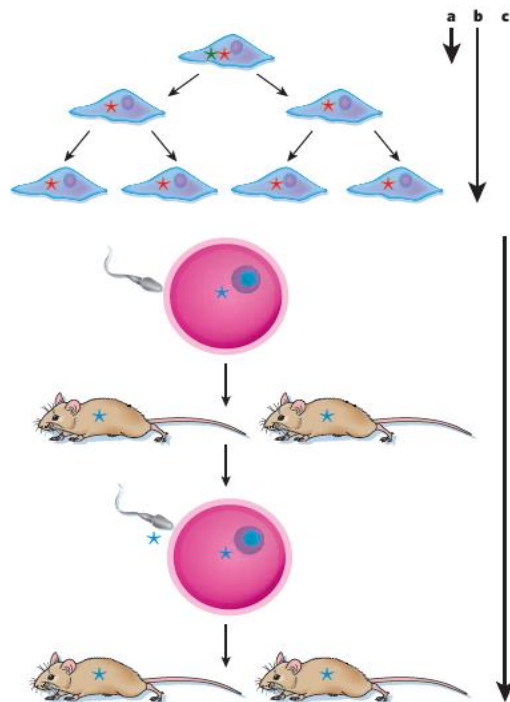


Figure 11 - Persistence of epigenetic marks. Alterations that last less than one cell cycle (green asterisk, a) do not qualify as epigenetic changes under the definition that strictly requires heritability, whereas non-mutational changes that are transmitted from one cell to its daughters (red asterisk, b) or between generations of an organism (blue asterisk, c) do qualify. Source: Bird (2007).

Transgenerational epigenetic inheritance was already found in several species, such as humans, mice, *Arabidopsis* and *Drosophila* (Cropley, Suter, Beckman, & Martin, 2006; Johannes et al., 2009; Morgan & Whitelaw, 2008; Pembrey, Saffery, & Bygren, 2014; Sen et al., 2015; Xing et al., 2007). In *Daphnia species*, transgenerational inheritance has been postulated but not confirmed so far (Agrawal, Laforsch, & Tollrian, 1999). In fact, several studies followed the effects of a range of environmentally relevant compounds for several generations of non-exposed *Daphnia* offspring (Vandeghehuchte, Kyndt, et al., 2009; Vandeghehuchte, De Coninck, Vandenbrouck, De Coen, & Janssen, 2010; Vandeghehuchte, Lemi re, et al., 2009; Vandeghehuchte, Lemi re, Vanhaecke, Berghe, & Janssen, 2010). The results of these works provided valuable insights as follows. DNA methylation levels in *D. magna* seem not to be affected by cadmium (Vandeghehuchte, Kyndt, et al., 2009). The exposure of one generation to a sub-lethal Zn concentration caused a reduction in the global DNA methylation levels of the offspring (F1), but this effect did not prevail in the second generation (F2) (Vandeghehuchte, De Coninck, et al., 2010; Vandeghehuchte, Lemi re, et al., 2009). Following daphnids' exposure to chemical substances known to affect DNA methylation in mammals (vinclozolin, 5-azacytidine, 2'-deoxy-5-azacytidine,

genistein and biochanin A), it was observed that only some, namely vinclozolin and 5-azacytidine, influence DNA methylation in *Daphnia*; both these chemicals provoked a reduction in the global DNA methylation levels of the offspring (F1) but this effect was only transferred to two subsequent non-exposed generations (F2) for the 5-azacytidine exposure (Vandeghechuchte, Lemière, et al., 2010).

Although it may be tempting to claim that these results indicate that epigenetic transgenerational inheritance indeed occurs in *Daphnia*, it should be noticed that the identification of transgenerational effects can be particularly challenging in species where the embryo develops in the body of the mother (e.g. *Daphnia* or humans). As Harris et al. (2012) stated “...*maternal exposure to environmental factors could affect the offspring either by retention of maternal epigenetic states in the germ line cells that give rise to the embryo, a true transgenerational effect, or more simply by exposure of the somatic cells of the embryo while it is in the mother*”. Therefore, these authors claimed that transgenerational epigenetic effects in *Daphnia* species can only be confirmed when epigenetic alterations persist through 3 post-exposure generations. In addition, Harris et al. (2012) and Vandeghechuchte et al. (2010) remarked that the examination of gene-specific methylation status would enhance the detection of transgenerational epigenetic effects by being more biologically informative. Nowadays, techniques such as bisulphite sequencing, methylated DNA immunoprecipitation (meDip) or DNA methylation sensitive restriction enzyme digests allow gene-specific analyses (Harris et al., 2012; Kurdyukov & Bullock, 2016).

1.7. Objectives and Structure of the Dissertation

The main aim of the present dissertation was to explore the potential role of epigenetic mechanisms in the evolutionary adaptation of freshwater species to climate change. The specific objectives addressed in the different chapters of the present document were:

- 1) to review the impacts of climate change on freshwater ecosystems and explore the evolutionary role of epigenetic mechanisms in both short- and long-term adaptation of freshwater organisms to the corresponding environmental fluctuations;

2) to explore the role of epigenetic transgenerational inheritance in the adaptation of *Daphnia magna* to increased levels of a selected model stressor reflecting climate change impacts in freshwater ecosystems, i.e. salinity.

In order to appropriately expose the work done while tackling these objectives, this dissertation was organized in **four chapters**. The present chapter (**chapter 1**) consists of a general introduction widely covering the main topics addressed throughout and recording on the global picture for a proper contextualisation of the following chapters.

Chapter 2 and **chapter 3** essentially contain two manuscripts already submitted or in preparation to be submitted for publication in international, peer-reviewed journals in the field. Chapter 2 consists of a theoretical review on the evolutionary role of epigenetic mechanisms, in a climate change context. Chapter 3 evaluates the importance of epigenetically acquired phenotypes and their transmission across several generations in the response of *Daphnia magna* exposed to high salinity levels.

Finally, **chapter 4** represents the final wrap-up of the major discussion items and provides the final remarks summarising the findings.

1.8. References

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Chapter 2 - The role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems: a review

2.1. Abstract

Freshwater ecosystems are amongst the most threatened ecosystems on Earth. Currently, climate change is the most important driver of freshwater transformation and its effects include changes in the composition, biodiversity and functioning of freshwater ecosystems. Understanding the capacity of freshwater species to tolerate the environmental fluctuations induced by climate change is critical to the development of effective conservation strategies. In the last few years, epigenetic mechanisms became increasingly considered in this context because of their pivotal role in gene-environment interactions. In addition, the evolutionary role of epigenetically inherited phenotypes is a relatively recent but promising field. Here, we revise and examine the impacts of climate change on freshwater ecosystems, exploring the potential role of epigenetic mechanisms in both short- and long-term adaptation of species. Focusing on the most promising future research avenues through the disentangling of the effects of climate change on freshwater biodiversity, potential molecular targets for epigenetic studies are specifically highlighted. Plus, available techniques and its adequacy, as well as the most suitable freshwater species to perform such studies are discussed.

Keywords: climate change, freshwater biodiversity, adaptation, epigenetic mechanisms, phenotype, transgenerational epigenetic inheritance

2.2. Introduction

Freshwater biodiversity is nowadays widely recognised as being severely threatened as a direct or indirect result of the human footprint. Thus, it urges to establish and maintain the effort towards monitoring freshwater biodiversity loss, unravelling the mechanisms involved and implementing mitigation and remediation measures. This is the general arena of this review. Its main aim was to collect evidences to provide valuable insights concerning the resilience of freshwater

species to cope with the environmental transformations induced by climate change, therefore supporting further establishment of protective measures against freshwater biodiversity loss. We present a global picture of the condition of freshwater resources and summarize the most significant drivers of freshwater transformation. The impacts of climate change-related stressors on freshwater ecosystems are extensively scrutinized and, in this context, the evolutionary role of epigenetic mechanisms is addressed by focusing on epigenetically determined phenotypes and their inheritance across several generations. Theoretical considerations and key experimental findings are presented, and an integrative discussion on these topics is made. Focusing on freshwater biota, future research avenues are highlighted, with key molecular targets to be tackled, the most current techniques available for the job and the most suitable freshwater model species being comprehensively discussed.

2.3. Condition of World's Freshwater: Drivers of Transformation

Hydrosphere is composed by all the available water (liquid, solid and gas) and its volume corresponds approximately to $1.386 \times 10^6 \text{ Km}^3$, but only 2.5% of it is actually freshwater. In terms of freshwater distribution, 68.7% is perennially frozen and 29.9% is groundwater (Carpenter, Stanley, & Vander Zanden, 2011; Shiklomanov, 2000). Moreover, only 0.26% of the Earth's freshwater can be found in lakes, reservoirs and rivers (Carpenter et al., 2011). Thus, freshwater is undoubtedly amongst the most precious resources for humankind. Besides humans' basic consumption needs, freshwater plays a key role in industrial processes, particularly in food, energy and fibre production, and it is vital to the dilution and degradation of discharged or deposited pollutants (Carpenter et al., 2011; Drechsel, Qadir, & Wichelns, 2015; Rockström, Gordon, Folke, Falkenmark, & Engwall, 1999). Consequently, freshwater scarcity has been set forward as one of the most dangerous threats for human societies (Carpenter et al., 2011; Mekonnen & Hoekstra, 2016).

Worldwide, human demand for freshwater currently approaches or exceeds its supply, and consequently, the provision of freshwater is only achieved following disruptive exploitation in numerous terrestrial and aquatic ecosystems (Corcoran,

2010; R. Jackson et al., 2001; Woodward, Perkins, & Brown, 2010). Plus, the drainage of chemicals from agricultural and urban areas and the discharge of contaminated waters from e.g. mining, medical and industrial activities lead to an increase in the quantity of exogenous chemicals such as organic compounds, metals, acids and alkalis, being released and spread throughout freshwater ecosystems (Carpenter et al., 2011; Durance & Ormerod, 2009; WWAP, 2017). Several of these contaminants are reportedly toxic to aquatic organisms and humans (Corcoran, 2010; Drechsel et al., 2015; WWAP, 2017). Besides direct anthropogenic impacts, climate change has been pointed out as another important driver of freshwater transformation (Carpenter et al., 2011; Michener, Blood, Bildstein, Brinson, & Gardner, 1997; Woodward et al., 2016; Woodward, Perkins, et al., 2010). It has risen as a consequence of human activities and its impacts can already be seen worldwide (IPCC, 2014; Mimura, 2013; Schewe et al., 2014).

In summary, freshwaters are among the most impacted and altered systems on Earth, although their protection and conservation is critical to the well-being of future generations (Carpenter et al., 2011; Dudgeon et al., 2006; WWAP, 2017).

2.4. Impacts of Climate Change on Freshwater Ecosystems

Climate change has provoked significant alterations in several climate trends (IPCC, 2014; Michener et al., 1997; Rahmstorf, 2007). These transformations are happening at an unprecedented pace and projections suggest that climate change will further cause profound alterations in freshwater systems within a few years (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Woodward, Dybkjær, et al., 2010; Woodward, Perkins, et al., 2010). Therefore, climate change has been touted the greatest emerging threat to global biodiversity and to the functioning of freshwater ecosystems (Bellard, Bertelsmeier, Leadley, Thuiller, & Courchamp, 2012; Dudgeon et al., 2006; Woodward, Perkins, et al., 2010). These ecosystems are already pressured by a myriad of anthropic stressors while they are heavily explored for goods and services (Carpenter et al., 2011; Foley, 2005; Karr & Dudley, 1981), and it is noteworthy that despite a small percentage of the Earth surface is covered by freshwater ecosystems (approximately 0.8%), they have

been estimated to support almost 6% of all described species (Dudgeon et al., 2006). In this way, the claim by Woodward et al. (2010) that “*freshwater biodiversity is disproportionately at risk on a global scale*” can be appropriately quoted. And indeed, climate change is transforming the composition, biodiversity and functioning of numerous freshwater ecosystems as synthesized in Table 1 (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Williams, 1987; Woodward et al., 2016; Woodward, Perkins, et al., 2010).

Table 1. Summary of the predicted and/or observed effects of climate change on the biological, chemical and physical features of freshwater ecosystems worldwide.

Reference	Assessment Region	Biological effects	Chemical and Physical effects
Hering et al., 2010	Europe	<ul style="list-style-type: none"> progressive loss of phytoplankton diversity; earlier phytoplankton Spring growth; suppression of large zooplankton species and consequent reduction in grazing intensity; enhanced macrophyte and algal growth; more complex life cycles in zooplankton species; earlier summer decline of daphnids; changes in the distribution of cold-water species; reduced reproduction success and increased predator pressure on the eggs and young life stages of cold-water species; extinction of fish and invertebrates in cold regions; extinction of cold stenothermic taxa in temperate and warm regions; increased colonization of small streams by alien species; alterations in fish communities, including in assemblages' structure; higher density of planktivorous cyprinid fish species; changes in the migration patterns of several fish species; greater proportion of terrestrial plant species in floodplain; lower benthic invertebrate abundance and diversity; influx of invasive vegetation species of wetlands; increased number of invertebrates r-strategists; changes in life strategies of bivoltine or semivoltine life cycles. 	<ul style="list-style-type: none"> higher water temperatures; reduced water quality; increased loss of water bodies by drying; changes in flow regimes, thus in sedimentation rates and channel morphology; higher differences of ice break-up timing between colder and warmer regions; higher frequency and duration of extreme events such as rainstorms and drought; increased sea-salt deposition; lower recharge of groundwater; more variation of carbon, phosphorus and nitrogen mineralisation rates; increased frequency of acidification pulses; higher runoff of total organic carbon; higher water oxygen depletion.
		<ul style="list-style-type: none"> changes in the structure and composition of aquatic communities; faster growth rates and smaller adult sizes in some freshwater invertebrates; earlier maturity of stream invertebrates; less food sources for stream insects; more frequent scouring of benthos; lower predation efficiency of certain fishes; increased parasite abundance; higher animal metabolic rates; lower genetic diversity of populations; increased likelihood of successful establishment of exotic aquarium escapees. 	<ul style="list-style-type: none"> warmer water temperatures, particularly in streams; drier flood plains; increased frequency of extreme climatic events, such as floods; increased eutrophication and oxygen depletion in lakes.

Allen, Smith, & Darwall, 2012	Indo-Burma Region (Thailand, Vietnam, Cambodia, Lao, Myanmar)	<p>severe impacts on species highly specialized, with complex life histories, with restricted ranges/limited distribution and/or with specific habitat requirements;</p> <p>increased migration of species that are relatively close to their upper thermal limits;</p> <p>increased migration to higher elevations or latitudes;</p> <p>loss of body mass in amphibians;</p> <p>higher metabolic rates in amphibians;</p> <p>shifts in the timing of fish migration;</p> <p>higher metabolic costs for fishes;</p> <p>increased invasion of alien species;</p> <p>higher transmission of fish parasites and diseases;</p> <p>skews in sex ratios of aquatic reptiles;</p> <p>depletion of plankton;</p> <p>food-web alterations.</p>	<p>higher water temperatures;</p> <p>less rainfall;</p> <p>increased saltwater intrusion and erosion in delta regions;</p> <p>lower water oxygen levels;</p> <p>exacerbation of anthropogenic stressors;</p> <p>more extreme flow events;</p> <p>higher tendency to develop dry-season droughts.</p>
Dallas & Rivers-Moore, 2014	South Africa	<p>changes in aquatic biodiversity;</p> <p>alterations in phenology and life-history patterns of several species;</p> <p>higher susceptibility of species with specialised habitat and/or microhabitat requirements, narrow environmental tolerances or thresholds, dependence on specific environmental triggers, dependence on interspecific interactions, and poor ability to disperse or colonise a new area;</p> <p>changes in the composition of communities;</p> <p>changes in species distribution and range;</p> <p>extinction of vulnerable species;</p> <p>higher number of invasive and pest species;</p> <p>higher number of waterborne and vector-borne diseases;</p> <p>changes in vegetation.</p>	<p>warmer water temperatures;</p> <p>increased frequency and intensity of extreme events;</p> <p>increased sedimentation and turbidity of the water;</p> <p>changes in nutrient cycles;</p> <p>higher transport of dissolved pollutants, such as pesticides and pathogens;</p> <p>mobilisation of adsorbed pollutants, such as metals and phosphorus;</p> <p>lower concentration of dissolved oxygen;</p> <p>higher salinization in semi-arid and arid areas;</p> <p>higher organic matter decomposition;</p> <p>changes in channel geomorphology;</p> <p>decreased longitudinal and lateral connectivity;</p> <p>changes in groundwater recharge rates.</p>
Mulholland et al., 1997	South-Eastern United States and Gulf Coast of Mexico	<p>simplified biological communities;</p> <p>favouring of species with short and multivoltine life histories;</p> <p>higher rates of primary production;</p> <p>less habitat for cool water species;</p> <p>expansion of subtropical species northwards;</p> <p>increased invasion of non-native species.</p>	<p>warmer water temperatures;</p> <p>lower water quality;</p> <p>higher frequency of short and strong rainfall events;</p> <p>increased organic matter decomposition;</p> <p>higher rates of nutrient cycling;</p> <p>changes in the salinity regimes and flushing rates of estuaries;</p> <p>shorter periods of inundation of riparian wetlands;</p> <p>exacerbation of anthropogenic stressors.</p>

Measuring and predicting the impacts of climate change on freshwater ecosystems has been the aim of several studies. Among them, warmer temperatures, greater variability of precipitation and higher water salinities have been highlighted as the most important derived stressors for freshwater ecosystems (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Dudgeon et al., 2006; Kefford et al., 2016). Warmer temperatures affect freshwater systems by decreasing the duration of the ice season, as they influence the melting of glaciers, permafrost and other ice sheets (Alley, 2000). Moreover, a warmer climate also contributes to changes in evaporation and precipitation ratios (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011). All these factors constitute important sources of limnological changes, since they provoke variations in the seasonality and magnitude of hydrologic income of lakes and streams (Carpenter et al., 2011; Fenoglio, Bo, Cucco, Mercalli, & Malacarne, 2010; Hering et al., 2010). The increase in the global surface temperature has also been leading to a rise in the heat absorbed by freshwater systems, especially on its surface (R. Jackson et al., 2001; Sabine et al., 2004). Warmer water temperatures decrease the amount of oxygen that can be dissolved, thereby decreasing the quantity of free oxygen that aquatic organisms can use in respiration (Carpenter et al., 2011; Hering et al., 2010; R. Jackson et al., 2001). Warmer waters also promote the decrease of habitat ranges of several aquatic organisms through their life cycle, the most iconic example being that of salmonids (Hoegh-Guldberg et al., 2007; Mohseni, Stefan, & Eaton, 2003; Schindler, 2001).

The rise in salinity is another climate change-related source of freshwater transformation (Mengel et al., 2016; Michener et al., 1997; Mimura, 2013; Rahmstorf, 2010). Mainly provoked by sea level rising, the rise in salinity observed in freshwater ecosystems has severe impacts on freshwater biodiversity, as it affects the survival and reproduction of several species which are typically poorly tolerant to variations in salts concentration (Carpenter et al., 2011; Gonçalves, Castro, Pardal, & Gonçalves, 2007; Martínez-Jerónimo & Martínez-Jerónimo, 2007; Mimura, 2013; Woodward, Perkins, et al., 2010). Even relatively small changes in salinity have been argued or shown to have an impact on freshwater and brackish ecosystems by depleting biodiversity and by changing their dynamics

and functioning (Cañedo-Argüelles et al., 2013; Kefford et al., 2016; Loureiro, Pereira, Pedrosa, Gonçalves, & Castro, 2013).

Once the salinity levels and/or temperature increases in a freshwater ecosystem, animals can only survive and reproduce if they adapt themselves to the new environment or if they behaviourally can avoid the stressor (Berg et al., 2010; Kefford et al., 2016; Nielsen, Brock, Rees, & Baldwin, 2003). However, freshwater ecosystems, lentic and semi-lotic ones in particular, are usually relatively isolated and physically fragmented within large terrestrial landscapes (Dudgeon et al., 2006; Woodward, Perkins, et al., 2010). Thus, freshwater species inhabiting these freshwater ecosystems cannot easily migrate or disperse into a new habitat while the environment is changing (Woodward et al., 2016; Woodward, Perkins, et al., 2010). Because of this decreased likelihood of a successful escape, freshwater species must adapt or will perish when facing the environmental fluctuations induced by climate change (Dudgeon et al., 2006; Eros & Grant, 2015; Woodward, Perkins, et al., 2010). Therefore, studying the adaptive potential of freshwater species is essential to understand the effects of climate change on these animals and their ecosystems (Heino, Virkkala, & Toivonen, 2009; Loureiro, Castro, Cuco, Pedrosa, & Gonçalves, 2013; Woodward et al., 2016; Woodward, Perkins, et al., 2010). More precisely, a deep understanding on the strategies used by freshwater organisms to cope with environmental change is critical to better picture the resilience of freshwater ecosystems under such environmental pressure, and hence for a conscientious development of effective protection/management actions towards the conservation of these valuable resources (Dudgeon et al., 2006; Loureiro, Castro, et al., 2013; Woodward et al., 2016; Woodward, Perkins, et al., 2010).

2.5. Epigenetic Mechanisms and their Evolutionary Role

Epigenetic mechanisms consist of potential heritable changes in gene activity, function or expression without altering the underlying DNA sequence (Bernstein, Meissner, & Lander, 2007; Bird, 2007; Goldberg, Allis, & Bernstein, 2007; Jaenisch & Bird, 2003). Epigenetic mechanisms include (i) the chemical modification of cytosine residues of DNA (DNA methylation); (ii) the modification of

histones associated with DNA (protein modifications) as they respond to environmental changes constraining chromatin structure and DNA accessibility (Bird, 2002, 2007; Goldberg et al., 2007; Kouzarides, 2007); (iii) non-coding RNAs (ncRNAs) that can control gene expression, forming complex RNA regulatory networks of the genome (Klimenko, 2017; Mattick & Makunin, 2006; Rinn & Chang, 2012). Epigenetic modifications are affected by environmental changes and are considered crucial for interpreting the genomes under the influence of physiological factors (Baccarelli & Bollati, 2009; Feil & Fraga, 2012; Ho & Burggren, 2010; Jaenisch & Bird, 2003).

Epigenetics has been increasingly gaining relevance in several fields, mainly due to studies revealing links between epigenetic mechanisms and several key molecular and cellular processes, such as transcriptional silencing, chromosome inactivation, transposable element regulation, development and tumorigenesis (Kingston, Tamkun, Baulcombe, & Dean, 2014; Morey & Avner, 2010; Sadikovic, Al-Romaih, Squire, & Zielenska, 2008; Sharma, Kelly, & Jones, 2009; Slotkin & Martienssen, 2007). One such field is evolutionary biology (Jablonka & Lamb, 2007; K. N. Laland et al., 2015; Pigliucci & Muller, 2010); while it is commonly assumed that the molecular basis of the evolutionary processes are random mutations followed by Natural Selection (Avice & Ayala, 2009; Jablonka & Lamb, 2007), these processes fail to explain several evolutionary phenomena (Burggren, 2016; Jablonka & Lamb, 2007; K. N. Laland et al., 2015). For example, they fail to explain some cases of rapid adaptation that are observed in natural populations because they are based on the progressive accumulation of small genetic and phenotypic differences (Bernardi & Bernardi, 1986).

Numerous environmental factors have been shown to influence epigenetic mechanisms (Blake & Watson, 2016; Guillelte, Parrott, Nilsson, Haque, & Skinner, 2016; Willbanks et al., 2016). As epigenetic mechanisms regulate gene expression, the consequence of a given environmental stress scenario can be a change in the phenotype (Burggren, 2016; Jaenisch & Bird, 2003; Moore, Le, & Fan, 2013). Moreover, epigenetic alterations are cleared and re-established at each generation, but some of them can be inherited through successive generations even when the initial stress pressure disappears (Bird, 2007; Daxinger

& Whitelaw, 2010; Vandegehuchte & Janssen, 2014). By this means, environmentally induced epigenetic modifications and their resulting phenotypes can be inherited through successive generations (Bird, 2007; Bräutigam et al., 2013; Burggren, 2016). Burggren (2016) highlighted that epigenetically inherited phenotypes work in the same sense as genetically inherited phenotypes, since they can be neutral, advantageous or disadvantageous for the organisms from the Natural Selection perspective. Therefore, epigenetically inherited phenotypes can also impact the overall fitness of the organisms that carry such traits and if advantageous, they can increase their adaptive capacity (Bossdorf, Richards, & Pigliucci, 2008; Burggren, 2016; Jaenisch & Bird, 2003; Varriale, 2014). Despite this similarity, there are significant differences between epigenetically determined phenotypes and genetically determined phenotypes, the most functionally dramatic being the fact that, when there are environmental fluctuations, epigenetically determined phenotypes arise more rapidly and far more broadly than genetically determined phenotypes (Burggren, 2016; Ho & Burggren, 2010; Jablonka & Lamb, 2007; Kussel & Leibler, 2005; Rando & Verstrepen, 2007).

In addition, it is well established that DNA methylation is an important facilitator of genome mutation and it has been shown that the rates of base mutation are remarkable higher in methylated cytosines than in non-methylated cytosines (Cooper & Krawczak, 1989; Coulondre, Miller, Farabaugh, & Gilbert, 1978; Denissenko, Chen, Tang, & Pfeifer, 1997; Nabel, Manning, & Kohli, 2012). Interestingly, Qu et al. (2012) showed that CpG methylation is a major determinant of proximal natural genetic variation. In fact, most individuals within a given natural population usually experience exposure to an environmental stressor at the same time and in the same extent (Burggren, 2016). When a group of organisms is exposed to a certain stressor, the same epigenetically determined phenotypes are known to be consistently acquired (Feil & Fraga, 2012; Klironomos, Berg, & Collins, 2013; Rando & Verstrepen, 2007), confirming that a given environmental stressor can thrive into the same epigenetic modifications (and their resulting phenotypes) in the different exposed organisms (Burggren, 2016; Weyrich et al., 2016). Furthermore, the analysis of DNA methylation in wide-ranging taxa revealed that the patterns of DNA methylation are conserved across deep

phylogenies (Mendizabal, Keller, Zeng, & Yi, 2014; Sarda, Zeng, Hunt, & Yi, 2012; Suzuki & Bird, 2008); and the genomic regions that reflect divergence of DNA methylation between related species seem to be enriched for both tissue and development specializations (Hernando-Herraez et al., 2013; Mendizabal et al., 2014; J. Wang, Cao, Zhang, & Su, 2012).

Nowadays, the evolutionary role of epigenetic mechanisms is a major and popular topic of scientific discussion. Several authors have been embracing the idea that the framework of the Modern Synthesis needs to be extended (Jablonka & Lamb, 2007; K. Laland et al., 2014; Pigliucci & Muller, 2010). Furthermore, it has been suggested that epigenetic mechanisms are important to the speciation process by enlarging the range of phenotypes available for the action of Natural Selection and for increasing the speed of speciation (Bossdorf et al., 2008; K. Laland et al., 2014; Mendizabal et al., 2014; Schrey, Richards, Meller, Sollars, & Ruden, 2012). Other specific fields linking to evolutionary biology to explain the natural dynamics of species, namely ecology, ecotoxicology and conservation biology, can also benefit from a better understanding of epigenetic mechanisms (Allendorf, Hohenlohe, & Luikart, 2010; Bossdorf et al., 2008; Mendizabal et al., 2014; Vandegehuchte & Janssen, 2011, 2014). As an illustration, the study by Vogt et al. (2015) stressed the potential importance of epigenetic mechanisms in the establishment of the freshwater marbled crayfish as a new species (*Procambarus fallax* f. *virginalis*), but epigenetic modifications are also important for the rapid adaptation of invasive species to different environments (Ardura, Zaiko, Morán, Planes, & Garcia-Vazquez, 2017; Chown et al., 2015; Pu & Zhan, 2017). Climate change is fostering the expansion of invasive species worldwide, these being recognised as one of the most dangerous threats to freshwater biodiversity and ecosystems (Table 1; Allen, Smith, & Darwall, 2012; Fenoglio, Bo, Cucco, Mercalli, & Malacarne, 2010; Friberg, 2014). It is indeed likely that by better exploring the role of epigenetic mechanisms in the adaptation of invasive species to freshwater habitats enables or supports the development of better and more efficient management strategies.

The summarized findings concerning epigenetically modified phenotypes, and the transmission of epigenetic marks across generations, support new

perspectives in the study of species adaptation to climate change towards a comprehensive understanding on the phenomenon. In this context, studies exploring the link between epigenetic inheritance, phenotype determination and Natural Selection are those more immediately required in order to clarify the evolutionary role of epigenetic mechanisms. Empirical data deriving from such studies are indeed crucial to build feasible new theoretical considerations in the field of evolutionary biology.

2.6. Epigenetic Adaptation to Climate Change

In a climate change context, extreme climatic events, the exacerbation of existing pollution and alien species invasion, water temperatures, greater variability in precipitation patterns and higher levels of salinity have been set forward as the most important threats to freshwater biodiversity and ecosystems (Bush & Hoskins, 2017; Cañedo-Argüelles et al., 2013; Carpenter, Stanley, & Vander Zanden, 2011; M. Jackson, Loewen, Vinebrooke, & Chimimba, 2016; Markovic, Carrizo, Kärcher, Walz, & David, 2017; Woodward et al., 2016; Woodward, Dybkjær, et al., 2010; Woodward, Perkins, & Brown, 2010). Focusing on temperature and salt stress, several studies explored the role of epigenetic mechanisms in the short-term responses of different species. Kumar & Wigge (2010) showed that the short-term adaptation of *Arabidopsis thaliana* to temperature changes is partly mediated through histone modification. Suter & Widmer (2013) found that the exposure of several generations of *A. thaliana* to increased salt concentrations and heat conditions induced heritable phenotypic changes, but interestingly, the heritable effects of the heat exposure disappeared in the second non-exposed generation. The exposure of a parthenogenetic population of *Artemia* to a non-lethal heat shock resulted in an increase in the levels of the heat shock protein 70, which configured increased tolerance to heat stress and additional resistance against the pathogenic bacteria *Vibrio campbellii* (Norouzitallab et al., 2014); the acquired phenotypic traits were transmitted to three successive non-exposed generations and correlated with altered levels of global DNA methylation and acetylated histones H3 and H4. Weyrich et al. (2016) investigated the adaptation of wild guinea pigs (*Cavia aperea*) to rising

temperatures. The authors exposed adult male guinea pigs (F0) to an increased ambient temperature and then compared the liver (as the main thermoregulatory organ) of the F0 fathers and F1 progeny, finding “an ‘immediate’ and ‘heritable’ epigenetic response” (Weyrich et al., 2016). Remarkably, Asselman et al. (2015) studied global cytosine methylation patterns following exposure of two different *Daphnia magna* genotypes to 15 stressor gradients and found that salinity was the single abiotic factor that significantly affected global DNA methylation levels in both clones tested. These studies support arguments on the decisive role that epigenetic mechanisms may have in constraining the response of freshwater populations to environmental fluctuations, particularly regarding stressor factors linked to climate change such as temperature and/or salinity.

Despite the interesting results obtained in the above-mentioned short-term studies, the role of epigenetic mechanisms in constraining responses in the long-term is particularly relevant in the context of this review since these are those cues allowing improved rationales in evolutionary arenas. Although more difficult to achieve, there are some reports in the literature in this context. Kronholm et al. (2017) manipulated DNA methylation and histone acetylation in the unicellular green alga *Chlamydomonas reinhardtii* both genetically and chemically. Through their method, the authors were able to monitor the amount of epigenetic variation generated or transmitted in populations adapting to three different challenging environments (salt stress, phosphate depletion, and high CO₂ levels) for two hundred asexual generations. They observed that, by reducing the amount of epigenetic variation available in the populations, a reduction of adaptation to the different environments occurs while the opposite would happen when levels of epigenetic variation were kept unchanged.

Varriale (2014) reviewed the evolutionary role of epigenetic mechanisms in vertebrate species, following on previous records on the variation of global DNA methylation levels of several cold- and warm-blooded vertebrates (e.g., (Jabbari, Cacciò, Païs De Barros, Desgrès, & Bernardi, 1997; Varriale & Bernardi, 2006a, 2006b). Curiously, the levels of methylation of the tested warm-blooded species were consistently lower than those of the cold-blooded vertebrates. Intrigued by these results, the authors latter showed that independently of the phylogenetic

distances, there was a negative correlation between methylation and temperature, when comparing the levels of 5-methylcytosine in the genome of fishes inhabiting different environments, namely polar, temperate and tropical regions (Varriale, 2014).

Artemov et al. (2017) investigated the role of DNA methylation in the adaptation of populations of the marine stickleback (*Gasterosteus aculeatus*) to freshwater conditions. Notably, the DNA methylation profile of marine sticklebacks transferred into freshwater partially converged to that of a freshwater stickleback, with the genes encoding ion channels (KCND3, CACNA1FB, and ATP4A) being differentially methylated between the marine and the freshwater populations. In agreement to these results, the potential importance of epigenetic mechanisms in constraining the evolution of K-strategist species has been stressed by Lighten et al. (2016), who compared the transcriptome of two recently diverged populations of the winter skate (*Leucoraja ocellata*) and found that epigenetic mechanisms determined the different profiles of gene expression observed in the populations; in addition, a relevant portion of the differentially expressed transcripts was correlated to genes whose function is involved in the different life-history traits of the populations.

Globally, the described findings seem to corroborate that epigenetic mechanisms indeed have an important evolutionary role. More precisely, some of these studies provide *in vivo* evidence that epigenetic mechanisms are capable of shaping the genome of organisms, allowing long-term adaptation of populations to environmental changes (Bernardi & Bernardi, 1986; Jablonka & Lamb, 2007; Wang, Crutchley, & Dostie, 2011). Particularly in a climate change scenario, there is strong evidence that epigenetic mechanisms contribute to the phenotypic plasticity and to the adaptive capacity of several organisms, including plants and fishes (Bossdorf et al., 2008; Bräutigam et al., 2013; Munday, 2014; Rey, Danchin, Mirouze, Loot, & Blanchet, 2016; Vannier, Mony, Bittebière, & Vandenkoornhuysen, 2015). Focusing on climate change, Rey et al. (2016) proposed a molecular engine that combines epigenetic mechanisms and transposable elements to explain how organisms can adjust their phenotypes, regulate the production of

phenotypic and genetic variation, and stably transmit the phenotypes across generations.

2.7. Conclusions and Perspectives for Future Research

It is becoming increasingly evident that epigenetic adaptation indeed is involved in the evolutionary adaptation of species to climate change. However, the adaptive potential of freshwater populations to climate change is still poorly understood, and both short- and long- term studies are needed to clarify whether epigenetic mechanisms contribute to the process (Allen et al., 2012; Kefford et al., 2016; Munday, 2014; Rey et al., 2016). To tackle this objective more promptly, one possible approach is to focus on the molecular mechanisms and cellular pathways that regulate the susceptibility of freshwater species to temperature and salinity fluctuations. For example and as to salinity fluctuations, the sodium pump, Na⁺/K⁺-ATPase, is the main responsible for osmoregulation in freshwater crustaceans, thus the genes encoding and regulating expression of this protein group are interesting candidate targets to look at in this context (Bianchini & Wood, 2008; Henry, Lucu, Onken, & Weihrauch, 2012; Latta, Weider, Colbourne, & Pfrender, 2012; Sáez, Lozano, & Zaldívar-Riverón, 2009). Moreover, the critical role of heat shock proteins in stress tolerance in general, and thermal stress in particular, has been highlighted for several freshwater species and marine invertebrates (Matthews, 2012; Solan & Whiteley, 2016). Thus, this set of genes and their regulators also seem promising candidates for future research regarding adaptation to climate change.

In addition, examination of gene-specific methylation status would enhance the detection of transgenerational epigenetic effects, which are more informative regarding adaptation strategies and the heritability driving evolution (Harris, Bartlett, & Lloyd, 2012; Vandegehuchte, De Coninck, Vandenbrouck, De Coen, & Janssen, 2010). Currently the tools for the job are available, and techniques such as bisulphite sequencing, methylated DNA immunoprecipitation (meDip) or DNA methylation sensitive restriction enzyme digests allow gene-specific analyses (Harris et al., 2012; Kurdyukov & Bullock, 2016).

Finally, the availability of well-known models for addressing epigenetics in the freshwater biota is pivotal to boost a better understanding of the responses to climate change by these ecosystems. In freshwater communities, *Daphnia* has been touted as an epigenetic model (Bell & Stein, 2017; Brander, Biales, & Connon, 2017; Harris et al., 2012). The underlying reason for this statement is the reproductive strategy of these animals, which allows the establishment of parthenogenetic clones with the consequent possibility of eliminating genetic variability in experimental trials (Harris et al., 2012; Weider & Hebert, 1987). Furthermore, the draft genome sequence of *D. magna* is now available, as well as a large quantity of ecological, ecotoxicological and evolutionary literature on *Daphnia* species that can facilitate the understanding of gene \times environment interactions (Baudo, 1987; Bell & Stein, 2017; Harris et al., 2012). Besides *Daphnia*, the marbled crayfish has been suggested as a suitable model organism for epigenetic, environmental epigenomics and evolutionary studies (Vogt, 2008). In fact, it has been used for experiments in different fields and bears the advantage of being a vigorous, clonable and eurytopic organism (Vogt, 2008, 2017; Vogt et al., 2015). The zebrafish (*Danio rerio*) seems also a suitable model organism for epigenetic research, particularly in development and disease studies (Detrich, Westerfield, & Zon, 2016; Martinez-Sales, García-Ximénez, & Espinós, 2015; Mudbhary & Sadler, 2011), although necessarily more representative of tropical ecosystems.

The studies addressed in this review have been helping to unveil the potential role of epigenetic mechanisms in the adaptation of species to climate change, over different temporal scales. Focusing on freshwater biodiversity, similar studies could provide valuable insights into the adaptive capacity of freshwater species to climate change, and mechanisms involved in their responses to habitat transitions. Therefore, they could be helpful resources for decision makers, being highlighted that the incorporation of transgenerational epigenetic heritability into risk assessment procedures could allow the establishment of protective measurements against biodiversity loss (Shaw et al., 2017). As climate change is contributing to the rapid decline of freshwater

ecosystems around the world, more than ever, their protection is an urgent and serious issue.

2.8. References

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Chapter 3 - Transgenerational effects of salinity stress in *Daphnia magna*: the role of epigenetic mechanisms

3.1. Abstract

The salinization of freshwaters is an important ecological issue. Salinity is a serious threat to freshwater ecosystems and an important form of environmental perturbation affecting freshwater biodiversity. Epigenetic transgenerational inheritance can influence the response of organisms to environmental stressors but this phenomenon remains barely explored in fields dedicated to the understanding of the effects of such stressors. An example of this knowledge scarcity is that for epigenetic responses in *Daphnia*, which is a key organism in aquatic toxicology, including within regulatory frameworks. We exposed one generation of *Daphnia magna* to high levels of salinity and found that the exposure provoked specific methylation patterns that were transferred to the three consequent non-exposed generations. These transgenerational effects were detected in four genes that encode for proteins (PAXIP1-associated glutamate-rich protein; DET1- and DDB1-associated protein; Prefoldin subunit 3; 60S ribosomal protein L36) that have important roles in the organisms' response to environmental change: DNA damage repair, cytoskeleton organization and protein synthesis. Our results highlight the potential importance of epigenetic transgenerational inheritance in the gene × environment interactions of *Daphnia*.

Keywords: Freshwater ecosystems, Epigenetic transgenerational inheritance, DNA methylation, Salinity, *Daphnia magna*

3.2. Introduction

Phenomena such as increased temperature, evaporation in waterbodies, and sea level rising have become more and more frequent in the past decade due to environmental change acceleration (IPCC, 2014). In this context, and also driven by secondary salinization referring to the accumulation of salts in both water and land ecosystems due to human activities (Cañedo-Argüelles et al., 2013; Griffith, Norton, Alexander, Pollard, & LeDuc, 2012), coastal freshwater ecosystems are

increasingly exposed to salinity fluctuations (Kefford et al., 2016; Schallenberg, Hall, & Burns, 2003). Experimental evidence has been generated on the potential impacts of salinity rising in freshwater communities, typically reflecting in reduced growth rates, development rates and fecundity, as well as increased mortality (Bailey et al., 2004; Hall & Burns, 2003; Jeppesen et al., 1994; Schallenberg et al., 2003; Stoks, Geerts, & Meester, 2014). Due to its intermediate position in trophic food web (Coldsnow, Mattes, Hintz, & Relyea, 2017; Lampert, 2006; Sommer & Stibor, 2002), zooplankton has been for long seen as a suitable and representative group to assess on such problematic, potentially indicating on putative effects of climate change on the structure and function of the whole ecosystem (Colbourne et al., 2011; Stoks et al., 2014)

A consistent decrease in the diversity and abundance of zooplankton communities exposed to increased salinity was reported by Hall & Burns (2003) and Schallenberg et al. (2003). Amongst freshwater zooplankters, cladoceran's responses to salinity fluctuations have been more widely studied. They resist to minor changes in salinity given their osmoregulatory capacity (Arnér & Koivisto, 1993; Martínez-Jerónimo & Martínez-Jerónimo, 2007), while they show differential physiological tolerances to several ionic components (Bos, Cumming, Watters, & Smol, 1996). *Daphnia* is a particularly important and well-studied cladoceran due to its central role in the freshwater food-web. *Daphnia* are hyperosmotic regulators, meaning that their body fluids have higher salt concentration than the water (Evans, 2005; Marshall, 2002) and denoting osmoregulation as an extremely important physiological process to assure the offsetting of the continuous ion loss to water (Aladin & Potts, 1995; Griffith, 2017; Lucu & Towle, 2003). The relevance of osmoregulation in *Daphnia* is even highlighted by specific features of their life-cycle. For example, since the brood chamber has direct contact with the external medium, it is imperative for the eggs to have an impermeable membrane until osmoregulatory organs are formed in the emerging juveniles (Aladin & Potts, 1995). A key actor in daphnids' osmoregulation is the transmembrane protein Na,K-ATPase, located at the basolateral membrane of mitochondria-rich cells (Sáez, Lozano & Zaldívar-Riverón, 2009). In fact, this protein is responsible for the active transport of sodium ions into the blood, whereas such ions enter cells

through a Na/H exchanger (Bianchini & Wood, 2008; Lucu & Towle, 2003; Madsen, Jensen, Tipsmark, Kiilerich, & Borski, 2007; Sáez et al., 2009; Tsai & Lin, 2007).

The response to such a new environmental challenge as saline intrusion requires that organisms adapt to the stressor, and they may cope with the change through different strategies. They may more immediately adapt by physiologically acclimating to the new conditions depending on phenotypic plasticity ranges (Bijlsma & Loeschcke, 2005; Boersma, Spaak, & De Meester, 1998; Castro, Consciência, & Gonçalves, 2007; Loureiro et al., 2015; Van Doorslaer et al., 2009). However, genotypic plasticity and concurrent microevolution may also mediate the tolerance of cladoceran populations to salinity in the long term (De Meester, 1996a, 1996b; Loureiro et al., 2012; Loureiro, Castro, Cuco, Pedrosa, & Gonçalves, 2013; Ortells, Reusch, & Lampert, 2005; Van Doorslaer et al., 2009; Van Doorslaer, Stoks, Jeppesen, & De Meester, 2007). Still concurring but yet remaining largely unexplored is the role of epigenetic mechanisms in adaptive strategies in general, and in particular that exhibited by cladocerans to increasing salinity stress. Yet, epigenetic mechanisms may be a crucial part of the adaptive and evolutionary response that can be transmitted to subsequent generations. To our best knowledge, the study by Asselman et al. (2015) is the single one so far addressing DNA methylation in *Daphnia magna* following short exposure of one generation to increased salinity levels, and the authors found that this was, amongst those stressors tested, the only abiotic stressor capable of affecting global DNA methylation levels.

Epigenetics comprises the study of both mitotically and meiotically heritable changes in gene activity and expression without a change in the DNA sequence (Bird, 2007; Goldberg, Allis, & Bernstein, 2007; Vandegehuchte & Janssen, 2011). Epigenetic modifications may be triggered by environmental factors, thus it is essential to understand how these can change organisms' response towards its ecophysiological context (Harris, Bartlett, & Lloyd, 2012; Vandegehuchte & Janssen, 2011). Several molecular mechanisms are known to be involved in epigenetics and new ones are continuously being discovered. However, the chemical modification of cytosine residues of DNA (DNA methylation) and the

modification of histones associated with DNA (protein modification) are those best described so far (Kouzarides, 2007; Lennartsson & Ekwall, 2009; Vandegehuchte & Janssen, 2011). These mechanisms can thereby regulate gene expression, and so they contribute to the determination of the phenotype of the organisms, contributing to the range of responses these organisms can show following exposure to environmental stressors.

In general, epigenetic alterations are cleared and re-established at each generation but some can be inherited through successive generations, sometimes even when the initial stress challenge is no longer present (Daxinger & Whitelaw, 2010; Skinner, 2009; Vandegehuchte & Janssen, 2011); since epigenetic alterations can be adaptive, selection for meiotic transmission may allow the transference of epigenetic information to the following generations (Harris et al., 2012). Although already documented in humans (Morgan & Whitelaw, 2008; Youngson & Whitelaw, 2008), mice (Cropley, Suter, Beckman, & Martin, 2006), *Drosophila* (Xing et al., 2007) and *Arabidopsis* (Johannes et al., 2009), this transgenerational inheritance is only postulated in *Daphnia*. The identification of transgenerational effects is indeed less straightforward in organisms in which the embryo undergoes development within the mother's body. Such a problem was faced by Guerrero-Bosagna et al. (2012) when investigating the existence of transgenerational effects associated with vinclozolin exposure in mice. In such a scenario, it is unclear whether progeny exposure could affect the offspring through the transmission of maternal epigenetic states (true transgenerational effect), or by the direct exposure of the somatic cells of the embryo to the stressor through the permeable brood pouch. In fact, embryos originating F1 and the germ line that then produces F2 are simultaneously present within the mother's body. Therefore, the epigenetic traits must persist until F3 to validate the existence of transgenerational inheritance, once this is the first unexposed generation and, consequently, the first generation in which a transgenerational phenomenon can be unequivocally concluded (Harris et al., 2012; Skinner, 2009).

Daphnids are relatively well-studied organisms regarding their ecophysiological responses to saline intrusion. This, along with the recognized role of *Daphnia* as an ecogenomic model (Miner, De Meester, Pfrender, Lampert, &

Hairston, 2012; J. R. Shaw et al., 2008), offers a unique opportunity to study genome-wide methylation patterns, gaining an insight on the importance of epigenetics in the adaptive responses of freshwater organisms to salinity fluctuations. *Daphnia magna* was used here following such a rationale. Moreover, *Daphnia magna* can be found in both fresh and brackish water habitats, in a range of water bodies that goes from large lakes to small ponds, some of them temporary, such as rock pools, through an originally Holarctic distribution that currently expanded to some arid climatic regions (Ebert, 2005; Vanoverbeke, De Gelas, & De Meester, 2007). As far as salinity tolerance is concerned, *Daphnia magna* is deemed euryhaline, surviving to salt ranges between 3.8 and 38 g.L⁻¹ (Alonso, 1996; Boronat, Miracle, & Armengol, 2001). As a cyclical parthenogen (Kleiven, Larsson, & Hobek, 1992), *Daphnia* can be experimented in such a way that genetic and epigenetic influences on organisms become clearly separated due to their clonal reproduction (Deng & Lynch, 1996; Harris et al., 2012; Koivisto, 1995), with the additional advantage of an easy handling through a short, highly productive reproductive cycle (Lampert, 2006).

Following on the above reasoning, the overall aim of the present study was to study whether epigenetic mechanisms may play a role in the adaptation of daphnids to freshwaters affected by saline intrusion. We specifically hypothesized that differential DNA methylation patterns can be identified in Na,K-ATPase genes and gene regulators, after challenging *Daphnia magna* with an increased salinity level and that these patterns are transmitted to subsequent generations. Further special attention was given to examining the possibility that gene-specific methylation patterns prevail through generations, with the ultimate objective of understanding whether there is a transgenerational inheritance of DNA methylation patterns in daphnids or not.

3.3. Material and Methods

3.3.1. Daphnia culturing

Monoclonal cultures of *Daphnia magna* (clone Beak) have been reared in our laboratory for more than 50 generations. Daphnids were cultured in ASTM hard water medium (ASTM, 1980) enriched with vitamins (Elendt & Bias, 1990) and

supplemented with an organic additive (Baird et al., 1989). Cultures were maintained under a constant temperature of $20 \pm 2^{\circ}\text{C}$ and a 16h^L:8h^D photoperiod (provided by cool fluorescent white lights). The culture medium was renewed and organisms were fed three times a week, with concentrated suspensions of *Raphidocelis subcapitata* (3×10^5 cells·mL⁻¹), which is cyclically cultured in Woods Hole MBL (Stein, 1973).

3.3.2. Exposure and sampling

Experiments were conducted under the previously described temperature and photoperiod conditions. Six cultures of 70 neonates (< 24 h old, collected from the 3rd-5th brood in bulk cultures) were established in plastic buckets filled with 4 L of test solution (57 mL per daphnid), three for each control (0 g/L NaCl) and salinity (4.1 g/L NaCl) exposures. The salt concentration used in the experiment was established on the basis of the results of a standardized short-term toxicity experiment to study the organisms' current sensitivity to NaCl (OECD, 2004). In this trial, different treatments (each with four replicates) were tested: 0, 3.50, 4.03, 4.63, 5.32 and 6.12 g/L NaCl. Five neonates, ageing less than 24 hours and born between the 3rd and 5th brood, were assigned to each replicate. The test was carried out in glass vials filled with 10 mL of test solution and the exposure lasted for 48 h, under a 16h^L:8h^D photoperiod and a temperature of $20 \pm 2^{\circ}\text{C}$, without food supply. Immobilization was recorded at the end of the test and EC_x values were estimated by Probit Analysis as a reference for setting the exposure level in the epigenetics experiment. Regression significance ($p < 0.05$) and significant goodness-of-fit (Pearson coefficient Chi-square statistics; $p < 0.05$) were confirmed, ensuring the feasibility of the estimates.

Regarding the epigenetics experiment, the exposure of daphnids lasted until the neonates initiating the experiment matured and reached their third brood. Subsequently, mothers were harvested, and stored at -80°C for DNA extraction, immediately after releasing the third brood, always with its brood pouch empty. All subsequent generations were started with 3rd brood new-born neonates (< 24h old) and maintained in clean medium until releasing their third brood, after which mothers were harvested and new generations established with the released

neonates (Figure 1). This procedure was repeated for all the four generations present in the experimental design.

DNA extraction was performed from daphnids' frozen tissue using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), according to manufacturer's instructions. A NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to roughly verify DNA quantity and quality. Quality criteria consisted of 260/230 ratios above 1.7, and 260/280 ratios between 1.8 and 2.1.

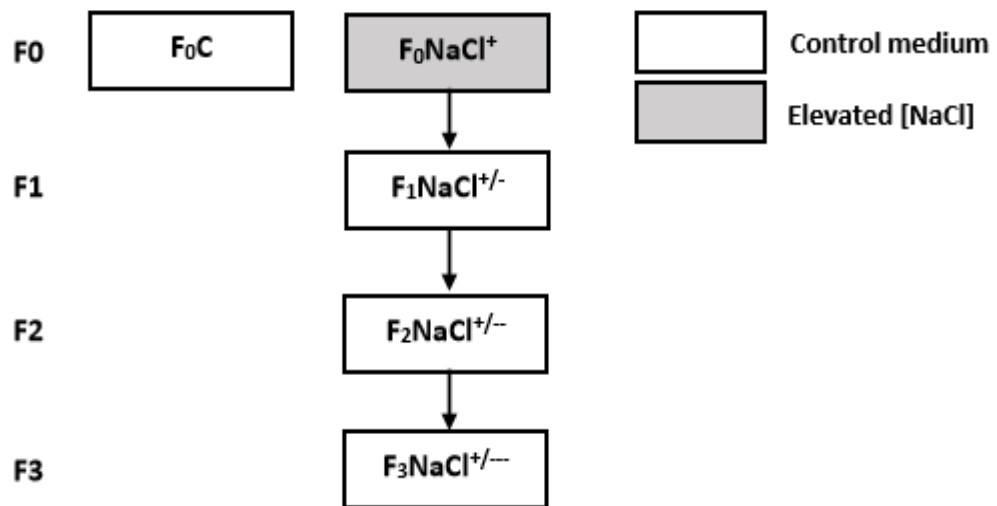


Figure 1 - Overview of the experimental design setup for the multigeneration experiment. F₀, F₁, F₂, F₃ represent generations. Arrows represent 3rd brood offspring. White rectangles represent clean medium while grey rectangles represent medium NaCl dissolved in the culture medium at 4.1 g/L. Plus and minus quoting immediately indicate the history of each culture regarding exposure to NaCl (+) and maintenance in clean medium (-).

3.3.3. Reduced representation bisulfite sequencing

Reduced representation bisulfite sequencing was performed over the DNA samples, i.e. the sampled DNA was digested with restriction enzymes, treated with bisulfite and finally sequenced (Meissner et al., 2005). These steps were carried out in the Ghent University reference center for next generation sequencing (NXTGNT) and allowed the detection and quantification of methylation profiles.

3.3.4. Bioinformatic analysis

Quality control of the raw data was executed with FastQC (version 0.11.5; Simon Andrews, Babraham Bioinformatics). Based on these results, the data was trimmed with Trim Galore, a wrapper tool around Cutadapt and FastQC (version 0.4.4; Felix Krueger, Babraham Bioinformatics), capable of automate quality and adapter trimming, as well as quality control, with an added functionality to remove biased methylation positions for RRBS sequence files. For our data, bases with a quality score below 30 were trimmed. Moreover, adapter sequences (Illumina) were removed and RRBS mode activated.

Subsequently, Bismark (version 0.18.1; Felix Krueger, Babraham Bioinformatics) was used for the mapping of our samples. The *Daphnia magna* genome assembly (Orsini et al., 2016, wfleabase.org) was used as a reference. Analysis of the data with Bismark resulted in methylation calls for every single cytosine analysed on each strand.

To determine the false positive rate, the error rate (sum of the non-conversion rate and T/C sequencing errors) was calculated. Results showed an average error rate of approximately 0.4% for our samples. Thus, to differentiate true positives from false positives, a model based on the binomial distribution $B(n, p)$ was used, in which n referred to the coverage depth of each potential methylated cytosine and p to the false positive rate. *P-value* was adjusted to 0.05 by Benjamini & Hochberg, 1995, hence only *P-values* < 0.05 were considered true positives.

Afterwards, bedtools intersect was used to identify the cytosines within genic regions. To this end, we used the gene models of Orsini et al. (Orsini et al., 2016).

3.3.5. Statistical analysis

Statistics were performed with R (R, Auckland, New Zeland). To quantify genes' methylation levels, the number of methylated cytosines was normalised to the total number of cytosines present in that gene. The existence of differentially methylated genes (DML) between treatments was assessed by DMLtest function for cytosines within genic regions, from DSS package (Park & Wu, 2016; Wu et al.,

2015). A Wald test was performed for each CpG site under the null hypothesis that the means of both treatments were equal. Subsequently, regions with statistically significant CpG sites were highlighted by callDML function. Furthermore, differentially methylated loci were sorted by statistical significance. For this test, alpha level was set up at $p=0.05$.

3.4. Results

The total number of non-significant and significant differentially methylated genes, in the comparisons between the control and the exposed and subsequent non-exposed generations, are represented in table 1.

Table 1. Total number of significant and non-significant methylated genes.

Comparison	Total number of genes	Total number of significantly differentially methylated genes ($p<0.05$)
Control – F0	20 450	93
Control – F1	18 334	96
Control – F2	23 944	84
Control – F3	23 193	103

A total number of 53 genes were exclusively differentially methylated between the control and NaCl-challenged organisms in the F0 generation, 60 in the F1, 31 in the F2 and 55 in the F3 (figure 2). Remarkably, four genes were significantly differentially methylated ($p<0.05$) in treated organisms compared to the control across the four generations (F0-F1-F2-F3), namely the genes that encode for the PAXIP1-associated glutamate-rich protein, the DET1- and DDB1-associated protein, the Prefoldin subunit 3 and the 60S ribosomal protein L36 (table 2). For all the comparisons (Control x F0, Control x F1, Control x F2 and Control x F3), these genes had higher methylation levels in the control than in the exposed and following non-exposed generations.

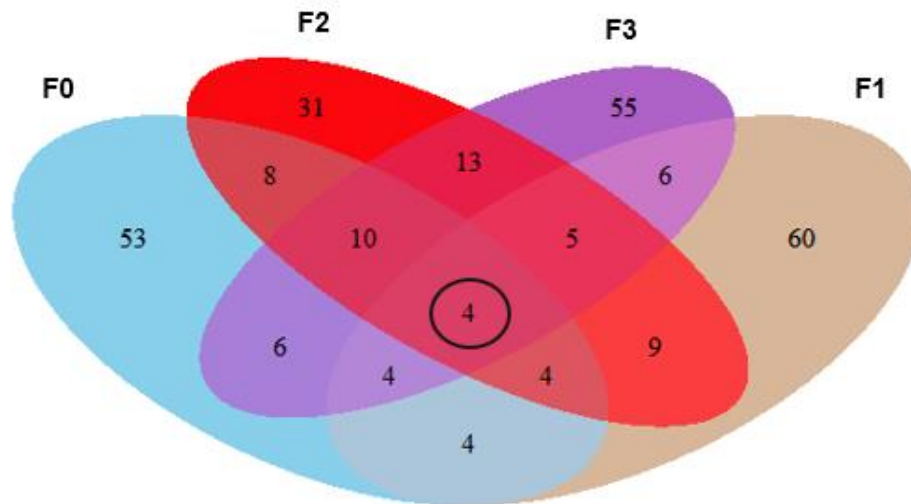


Figure 2 - Venn Diagram overlapping the genes differentially methylated between NaCl treatments and the control through the different generations ($p < 0.05$). A highlight was made to the four genes that were found differentially methylated across the four generations comprised in the experiment.

Four genes were significantly differentially methylated ($p < 0.05$) between NaCl-treated and the control across three consecutive generations (F0-F1-F2), namely the genes that encode for the Golgi apparatus membrane protein TVP23 B, the NADH dehydrogenase [ubiquinone] 1 beta subcomplex, the Small nuclear ribonucleoprotein G and an uncharacterized protein. For all the comparisons (Control x F0, Control x F1 and Control x F2), the genes that encode the Golgi apparatus membrane protein TVP23 B and the Small nuclear ribonucleoprotein G had higher methylation levels in the control than in the exposed and following non-exposed generations. However, the gene that encodes the NADH dehydrogenase [ubiquinone] 1 beta subcomplex showed higher methylation levels in the control than in the exposed and subsequent non-exposed generations.

Table 2. Significantly differentially methylated genes, across the four generations comprised in the experiment and the first three generations.

	Gene ID	Annotation	Comparison	p-value
Genes differentially methylated across F0/F1/F2/F3 generations	Dapma7bEVm 007142t1	PAXIP1-associated glutamate-rich protein	Control – F0	2.97e ⁻⁰³
			Control – F1	8.09e ⁻⁰⁶
			Control – F2	8.68e ⁻⁰⁴
			Control – F3	2.27e ⁻⁰³
	Dapma7bEVm 007954t1	DET1- and DDB1- associated protein	Control – F0	2.29e ⁻⁰⁷
			Control – F1	1.65e ⁻⁰⁸
			Control – F2	2.85e ⁻⁰⁴
			Control – F3	1.57e ⁻⁰⁵
	Dapma7bEVm 007536t1	Prefoldin subunit 3	Control – F0	5.07e ⁻⁰³
			Control – F1	4.19e ⁻⁰⁵
			Control – F2	2.33e ⁻⁰⁵
			Control – F3	3.22e ⁻⁰²
Genes differentially methylated across F0/F1/F2 generations	Dapma7bEVm 010667t1	60S ribosomal protein L36	Control – F0	1.22e ⁻⁰³
			Control – F1	3.63e ⁻⁰⁴
			Control – F2	2.70e ⁻⁰³
			Control – F3	1.38e ⁻⁰³
	Dapma7bEVm 001295t1	Golgi apparatus membrane protein TVP23 B	Control – F0	1.18e ⁻⁰²
			Control – F1	8.50e ⁻⁰³
			Control – F2	5.28e ⁻⁰³
	Dapma7bEVm 004966t1	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	Control – F0	1.87e ⁻⁰²
			Control – F1	3.00e ⁻⁰³
			Control – F2	3.50e ⁻⁰²
	Dapma7bEVm 028806t1	Small nuclear ribonucleoprotein G	Control – F0	1.18e ⁻⁰²
			Control – F1	2.49e ⁻⁰³
			Control – F2	3.74e ⁻⁰³
	Dapma7bEV m026583t1	Uncharacterized protein	Control – F0	4.53e ⁻⁰³
			Control – F1	1.70e ⁻⁰⁸
			Control – F2	5.62e ⁻⁰⁷

3.5. Discussion

The epigenetic repertoire in *Daphnia* includes not only DNA methylation but also histone modifications (Lai et al., 2016; Robichaud, Sassine, Beaton, & Lloyd, 2012). In addition, non-coding RNAs have been identified in *Daphnia pulex* and *Daphnia magna* (Chen, McKinney, Nichols, & Sepúlveda, 2014; Ünlü, Gordon, & Telli, 2015). Therefore, the potential importance of epigenetic mechanisms in the gene x environment interactions of *Daphnia* species has been receiving increased attention (Asselman et al., 2017; Bell & Stein, 2017; Brander, Biales, & Connon, 2017; Harris et al., 2012; J. L. A. Shaw et al., 2017). Particularly, several

alternative phenotypes have been postulated to be epigenetically determined and transgenerationally inherited (Bell & Stein, 2017; Harris et al., 2012).

For example, as daphnids are exposed to predator cues (kairomones) the formation of protective helmets occurs in both the exposed mothers and their non-exposed progeny (Agrawal, Laforsch, & Tollrian, 1999; Laforsch & Tollrian, 2004). Besides, the formation of neckteeth and environmental sex determination have been proposed to be examples of epigenetic inheritance (Harris et al., 2012). Several experimental studies followed the effects of a range of environmentally relevant compounds for several generations of non-exposed *Daphnia* offspring (Vandeghechuchte, Kyndt, et al., 2009; Vandeghechuchte, De Coninck, Vandenbrouck, De Coen, & Janssen, 2010; Vandeghechuchte, Lemièrè, & Janssen, 2009; Vandeghechuchte, Lemièrè, Vanhaecke, Berghe, & Janssen, 2010), with evidences suggesting a role of epigenetics in defining response patterns. The exposure of one generation to a sub-lethal Zn concentration caused a reduction in the global DNA methylation levels of the offspring (F1), but this effect did not prevail in the second generation (F2) (Vandeghechuchte, Lemièrè, & Janssen, 2009). Following daphnids' exposure to chemical substances known to affect DNA methylation in mammals (vinclozolin, 5-azacytidine, 2'-deoxy-5-azacytidine, genistein and biochanin A), it was observed that only vinclozolin and 5-azacytidine influence DNA methylation in *Daphnia*; both these chemicals provoking a reduction in the global DNA methylation levels of the offspring (F1) but this effect was only transferred to two subsequent non-exposed generations (F2) for the 5-azacytidine exposure (Vandeghechuchte, Lemièrè, et al., 2010).

Despite these promising results, environmentally induced epigenetic transgenerational effects need to be monitored through 3 post-exposure generations (Bell & Stein, 2017; Brander et al., 2017; Harris et al., 2012; J. L. A. Shaw et al., 2017). Following this rationale, our experimental design allowed us to confirm that epigenetic transgenerational inheritance indeed occurs in *Daphnia magna*. More precisely, we demonstrated that the exposure of one generation to high salinity levels provoked methylation patterns that were transferred to the three subsequent non-exposed generations. Remarkably, the transgenerational effects were detected in four genes that encode for important proteins (PAXIP1-

associated glutamate-rich protein; DET1- and DDB1-associated protein; Prefoldin subunit 3; 60S ribosomal protein L36) for the regulation of responses to environmental stress.

Actually, the PAXIP1-associated glutamate-rich protein is known to have multiple roles in gene regulation (Cho et al., 2007; Kumar et al., 2014; Z. Zhang, Sun, Cho, Chow, & Simons, 2013). It is part of the histone methyltransferase MLL2/MLL3 complex, which suggests its potential role in epigenetic transcriptional activation (Cho et al., 2007). In addition, this protein controls genome stability and it is crucial to DNA repair (Kumar et al., 2014). Salinity is known to provoke DNA breaks and the potential higher expression of the PAXIP1-associated glutamate-rich protein suggest its recruitment to repair the DNA damage induced by salinity (Demirkiran, Marakli, Temel, & Gozukirmizi, 2013; Dmitrieva, Cui, Kitchaev, Zhao, & Burg, 2011; Gong, Cho, Kim, Ge, & Chen, 2009). The DET1- and DDB1-associated protein is conserved and its orthologs can be found in vertebrates and invertebrates (Pick et al., 2007). This protein is involved in ubiquitination and subsequent proteasomal degradation of target proteins (Lee & Zhou, 2007; Pick et al., 2007). It is known to be part of the DDD-E2 complex which interacts with multiple ubiquitin E3 ligases, and CUL4A and WD repeat proteins (Irigoyen et al., 2014; Pick et al., 2007; Schumacher, Wilson, & Day, 2013). Under abiotic stress, ubiquitin/proteasome systems have been pointed out as key mechanisms to optimize the adaptation and survival strategies of different species to environmental stressors (Chiu, Pan, Zhao, & Gazzarrini, 2016; Jeon et al., 2012; Mudagandur, Gopalapillay, & Vijayan, 2016; Yu, Wu, & Xie, 2016). Prefoldin subunit 3 binds specifically to cytosolic chaperonin and has an important role in the functioning of the actin and tubulin-based cytoskeleton (Millán-Zambrano & Chávez, 2014; Rommelaere et al., 2001; Vainberg et al., 1998). In particular, it is critical to the tubulin complex assembly and it has been directly implied in the tolerance of *Arabidopsis* to salt stress (Gu et al., 2008; Rodríguez-Milla & Salinas, 2009). In fact, it has been suggested that the cytoskeleton plays an essential role in plant adaptation to high levels of salinity (Rodríguez-Milla & Salinas, 2009; Soda, Sharan, Gupta, Singla-Pareek, & Pareek, 2016; C Wang et al., 2010; Che Wang, Zhang, & Huang, 2011). Consistently, our results suggest that cytoskeleton

may also be important in the response of *Daphnia magna* to salinity. The differentially methylated 60S ribosomal protein L36 is a component of the large ribosomal subunit and it is relevant for protein synthesis (Alkayal et al., 2010; Koia, Moyle, Hendry, Lim, & Botella, 2013; Wei et al., 2017). Alkayal et al. (2010) studied the effects of salinity stress on the algae *Dunaliella salina* and showed that a big portion of the differentially expressed sequence tags (EST) (control vs. salinity treatment) were related to protein synthesis (the vast majority encoded ribosomal proteins). Under salt stress, organisms invest substantial resources in maintaining protein synthesis to improve their resilience (J. Wang et al., 2015; Wei et al., 2017; Zhu, 2002).

Four genes were also differentially methylated from the control in the exposed and two subsequent non-exposed generations (F0-F1-F2). These genes encode for proteins (Golgi apparatus membrane protein TVP23 B; NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial; Small nuclear ribonucleoprotein G; Uncharacterized protein) that are also potentially important in the response to salinity stress. These proteins are related to vesicular trafficking, mitochondrial membrane respiratory chain and splicing of cellular pre-mRNAs, and there are several studies that highlighted the relevance of these molecular processes and pathways in the response of different species to abiotic stressors in general and salinity in particular (Brandizzi, Snapp, Roberts, Lippincott-Schwartz, & Haves, 2002; Ding et al., 2014; Gallois et al., 2009; Huang et al., 2011; Ma et al., 2015; Sanan-Mishra, Tuteja, & Sopory, 2002; F. Zhang et al., 2016).

Our results suggest that DNA methylation is not involved in the regulation of the expression of the Na,K-ATPase subunits in salt stress conditions. However, the gene that encodes for the V-type proton ATPase subunit G was differentially methylated between NaCl-challenged organisms and the control in the F0, F2 and F3 generations, revealing that this gene may be epigenetically regulated. Under stress conditions, such as salinity, the survival of cells strongly depends on maintaining or adjusting the activity of the V-ATPase (Beyenbach & Wieczorek, 2006; Toei, Saum, & Forgac, 2010). For a better understanding of the importance of epigenetic mechanisms in *Daphnia* gene x environment interactions and in the

regulation of the expression of the Na,K-ATPase subunits, the correlation between different methylation levels and differential gene expression and protein levels should be established. In addition, the role of other epigenetic mechanisms, such as histone modifications, should be further explored (Harris et al., 2012; Lai et al., 2016; Robichaud et al., 2012).

The present study supports the recent claims that epigenetic inheritance should be incorporated into risk assessment procedures, as due to epigenetic transgenerational inheritance environmental perturbations can influence the future health and function of the ecosystems (Mirbahai & Chipman, 2014; J. L. A. Shaw et al., 2017; Vandegehuchte, Kyndt, et al., 2009; Vandegehuchte, Lemière, Janssen, et al., 2009; Vandegehuchte & Janssen, 2011). Globally, our results suggest that DNA methylation is an important mechanism in the response of *Daphnia magna* to high levels of salinity. In addition, the inheritance of DNA methylation patterns in non-exposed generations reinforces the potential importance of epigenetic transgenerational inheritance in the responses of *Daphnia* species to environmental changes.

3.6. References

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Chapter 4 - General discussion and final remarks

Throughout this dissertation, the importance of epigenetics in the response of freshwater organisms to the environmental transformations induced by climate change has been extensively explored.

On a global scale, climate change is altering the composition, biodiversity and functioning of numerous freshwater ecosystems. Sea level rise is one of the most important climate change-related consequences as it promotes the salinization of freshwater ecosystems. In fact, saline intrusion is recognized as an important form of environmental perturbation to freshwater biodiversity.

Epigenetic mechanisms are crucial to better understand gene \times environment interactions. Environmental factors influence epigenetic mechanisms, therefore contributing to the determination of phenotypes. Furthermore, epigenetically acquired phenotypes have been proven to be inherited across several generations, although this is not widely demonstrated experimentally.

Daphnia have a key role in freshwater ecosystems, being used as indicators of environmental change in several fields, such as ecology, ecotoxicology and evolutionary biology. Moreover, *Daphnia* has been set forward as an epigenetic model organism and its ecophysiological response to salinity intrusion is well characterized. Consequently, *Daphnia* species are ideal to detail the study of the response of freshwater organisms to environmental salinity fluctuations.

This dissertation unveiled that epigenetic mechanisms have an important evolutionary role by contributing to the phenotypic plasticity and to the adaptive capacity of different taxonomic groups. Remarkably, epigenetic mechanisms seem to allow both short- and long-term adaptation of freshwater populations to the environmental transformations induced by climate change. Another key finding of this work was that a one-generation exposure of *Daphnia magna* to high salinity levels resulted in an epigenetic adaptation to the hyperosmotic environment. More precisely, the exposure provoked DNA methylation patterns that were transferred to three subsequent non-exposed generations. In addition, these transgenerational effects were observed in genes that encode for proteins with key roles (DNA damage repair, cytoskeleton organization and protein synthesis) in the response of different organisms to environmental change.

The mentioned findings are novel and critical to better picture the resilience of freshwater species under environmental change. In addition, such results open future research avenues. Amongst them is included the understanding of the role of other epigenetic mechanisms (e.g. histone modifications and non-coding RNAs) in the gene \times environment interactions of freshwater species. Besides, to understand if temporary exposures to environmental stressors affect the future health and function of the ecosystem through epigenetic transgenerational inheritance is a key research topic.

In the future, the exploration of the mentioned far-reaching results and proposed research questions will certainly have important implications in several fields such as ecotoxicology, ecology, aquatic toxicology and evolutionary biology, and also more robust conclusions on whether epigenetic inheritance should be considered in higher-tier environmental risk assessment frameworks as a valuable indicator will certainly arise.